"DESIGNER YEAST," A NEW REAGENT FOR ENANTIOSELECTIVE BAEYER-VILLIGER OXIDATIONS

By

KIETH WILLIAM REED

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

1996

This work is dedicated to Dr. William A. Reed

ACKNOWLEDGMENTS

I would like to thank my wife Mary for her moral support, the U.S. Army for the opportunity to participate in the Advanced Civil Schooling program and for the financial support, Dr. Dolbier for the loan of a GC to get this work off-the-ground, Micheal Bartberger for his help in GC matters, the Stewart Group, Dr. Margaret Kayser, Kayser Group for synthesis of the 2-substituted starting material, and finally, Dr. Jon Stewart for his guidance and patience.

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Abstract of Thesis Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Master of Science

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May, 1996

Chairman: Dr. Jon D. Stewart
Major Department: Chemistry

The catalytic repertoire of baker's yeast has been expanded to include enantioselective Baeyer-Villiger oxidations. To create this catalyst, the *Acinetobacter* sp. (NCIB 9871) cyclohexanone monooxygenase gene was cloned into a yeast expression plasmid and this vector was used to transform baker's yeast (*Saccharomyces cerevisiae*). Whole cell-mediated Baeyer-Villiger reactions were carried out on a 1.0 mmole scale and several cyclic ketones were converted in 20-30 hours into the corresponding lactones in isolated yields of 54-83%. Under our reaction conditions, ketone reduction caused by the host yeast reductases constituted only a minor side-reaction. In the first phase of this work, reaction conditions for the Baeyer-Villiger yeast-mediated oxidation of cyclohexanone were optimized. The second phase

involved the oxidation of prochiral 4-substituted cyclohexanones by our engineered yeast. The enzyme-mediated oxidation of these substrates afforded lactones with very high enantioselectivities. While we did encounter some problems with substrate solubilities, these were easily solved by the addition of stoichiometric amounts of β-cyclodextrin. In the third and final phase, we studied the oxidation of racemic 2-substituted cyclohexanones. As the size of the substituent increased, we found that the kinetic resolutions improved dramatically. In all cases, the (*S*)-ketone was more reactive than the antipode. This kinetic resolution allowed us to isolate both enantiomerically enriched ketones and lactones, generally with ee values of greater than 98%. Recovered yields of the lactone products varied from 54 to 79%. In conclusion, we have created a simple reagent capable of performing a chiral Baeyer-Villiger oxidation. The use of this reagent requires little or no training in biochemistry or microbiology and hence would be very useful to all synthetic chemists.

CHAPTER 1
BACKGROUND

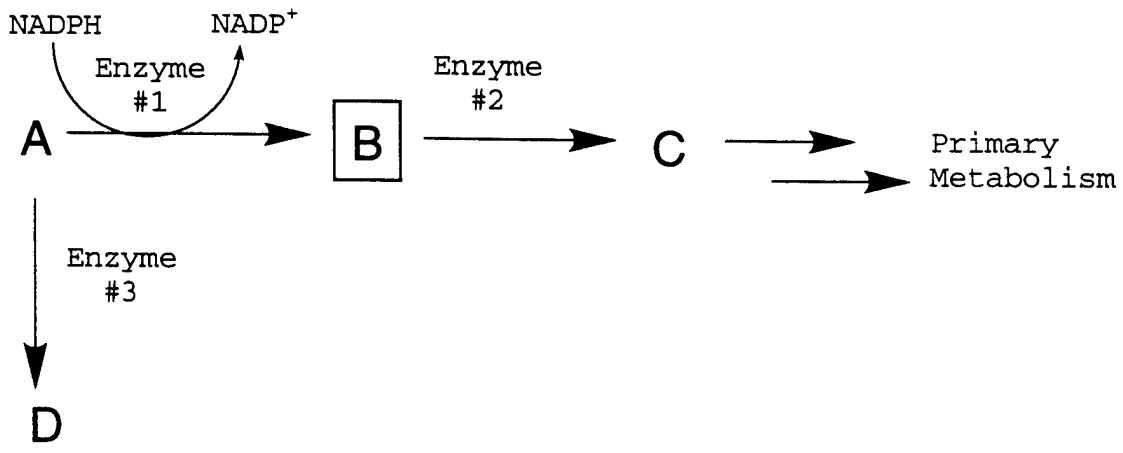
1.1 Introduction

The focus of this research has been to produce a model system for the expression of synthetically useful enzymes in baker's yeast. Baker's yeast (*Saccharomyces cerevisiae*) has been extensively used by chemists in the past, principally to catalyze enantioselective reductions (Fuganti et al., 1990; Kitahara et al., 1994; Servi et al., 1990). It has proven to be very competitive with standard synthetic methods and is easily handled and stored. Given this ease of use, we feel that the expansion of possible synthetic reactions that can be catalyzed by baker's yeast would be of great benefit to organic chemists.

1.2 Whole Cell and Purified Enzymes in Organic Synthesis

General. Enzymes have been shown to have enormous potential for organic synthesis (Crout & Christen, 1989; Davies et al., 1989; Faber et al., 1995; Santaniello et al., 1992). Until recently, the use of enzymes for organic synthesis has generally been accomplished by two methods, each of which has its own advantages and limitations. In the

whole-cell strategy, the substrate is added to a growing culture of a microorganism that expresses the desired enzyme. After the chemical reaction has occurred, the products are purified away from the cell mass. The second approach utilizes purified enzymes, obtained from either the microorganism or tissue in which it naturally occurs, or after overexpressing the enzyme in *Escherichia coli*. In this case, the substrate is incubated in the presence of the purified enzyme along with any necessary cofactors. The latter can be supplied in either stoichiometric amounts or in catalytic quantities if a cofactor regeneration system is employed (see, for example Abril et al., 1989). Before considering specific enzymes, it is useful to consider the strengths and weaknesses of the two strategies. Scheme 1.1 illustrates a hypothetical metabolic pathway that might occur in a microbial cell. B, the desired product, is produced by enzyme #1 in an NADPH-dependent reaction.



Scheme 1.1 Hypothetical Metabolic Pathway

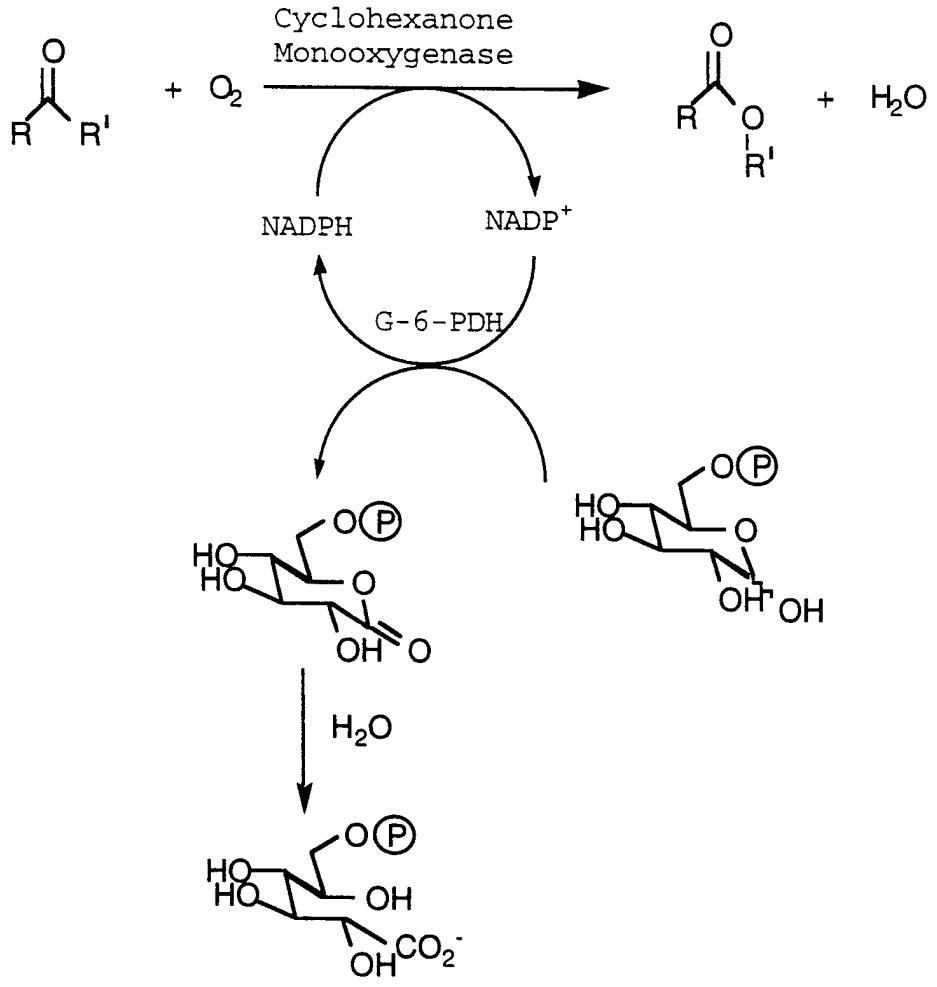
Whole cell systems. There are two major advantages to using whole microbial cells to convert A into B. First, cofactor synthesis and regeneration ($NADPH/NADP^+$) is performed by the cell. Secondly, it is relatively simple to grow the microorganism in the presence of substrate A to obtain the desired product. However, while whole cell biotransformations look appealing, there are several limitations to their use. 1) The enzyme of interest (enzyme #1) is part of a metabolic pathway. Hence product B may be further metabolized by other enzymes (represented here as enzyme #2) in the cell. 2) It is also possible that substrate A can be metabolized by other cellular enzymes to product D (represented here as enzyme #3). 3) Some microorganisms are pathogenic, which introduces restrictions on their use and availability. 4) It is difficult to predict which microorganism will be appropriate for each substrate, meaning that one must screen a large number of candidates to find the correct microorganism. This limitation necessitates expertise in handling varied microorganisms within a lab.

Purified enzyme systems. The hypothetical metabolic pathway shown in Scheme 1.1 also illustrates the strengths and weaknesses of using purified enzymes for synthesis. In this case, purified enzyme #1 would be used to convert A into B. There are three major advantages in this approach. 1) The problems of competing enzymes for A and B are eliminated. 2) Toxicity effects of the substrate or product on the microorganism are avoided. 3) Only limited expertise

in handling microorganisms is necessary. However, the isolated enzyme strategy also introduces several problems. 1) Purified enzymes can be expensive and often have a limited shelf life. The source of many potentially useful enzymes is vertebrate tissue, which can be expensive and difficult to obtain. 2) Individual labs must spend a great deal of time and effort to purify and sometimes immobilize the enzymes. 3) Purified enzyme systems often require cofactors such as NADPH. These can be extremely expensive, although NADPH can be regenerated in *situ* using glucose-6-phosphate and glucose-6-phosphate dehydrogenase (G-6-PDH) from *Leucoucstoe mesenteroides* (Scheme 1.2)(Abril, et al., 1989).

Recombinant yeast systems. To combine the strengths of both the whole-cell and isolated enzyme approaches, we have explored a new strategy. In this system, which we refer to as "designer yeast," we engineer baker's yeast to express a foreign gene whose protein product performs useful synthetic chemistry. This allows us to use whole cells of the engineered yeast for simple biotransformations. There are several advantages to this system. 1) By only expressing one enzyme from a metabolic pathway, we minimize the possibility that the product will be further degraded by the host organism. This allows one to predict the reaction product and provides almost all of the benefits of purified enzyme systems. 2) While this approach has been explored using *E. coli*., (Draths et al., 1994; Herrmann et al., 1994), yeast

has been used much more extensively by organic chemists, and demands little microbial expertise and specialized equipment.



Scheme 1.2 NADPH Regeneration (Abril et al, 1989)

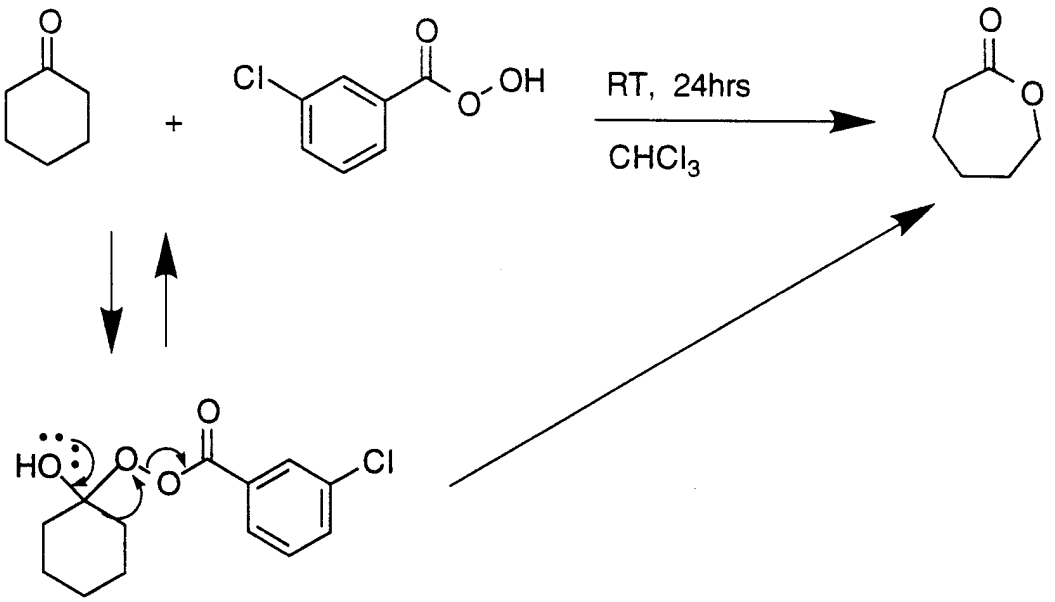
3) The yeast expression system eliminates the requirement for cofactor regeneration by creating a self-contained factory in which cofactors are provided and regenerated by the host organism. 4) Due to its eukaryotic nature, yeast is better suited for expressing vertebrate enzymes than *E. coli*. 5) In principle, it should be possible to express more than one

heterologous enzyme in yeast simultaneously, which should allow one to create novel metabolic pathways. To prove the feasibility and demonstrate the advantages of this approach, we have expressed a bacterial enzyme that catalyzes enantioselective Baeyer-Villiger oxidations on a broad array of substrates. In this way, we have created the baker's yeast equivalent of a chiral peracid.

1.3 Chemical vs Biological Baeyer-Villiger Reactions

Chemical Baeyer-Villiger reactions. The oxidation of ketones to esters and lactones using the Baeyer-Villiger reaction is a useful and reliable synthetic reaction (Hassall, 1957). Unfortunately, simple reagents for performing enantioselective Baeyer-Villiger oxidations are in an early stage of development (Bolm et al., 1993, 1994, 1995). The Baeyer Villiger reaction is generally accomplished using hydrogen peroxide in weakly basic solutions or an organic peracid, such as peroxyacetic, trifluoroperoxyacetic, monoperoxyphthalic acids or *m*-chloroperoxybenzoic acid (*m*-CPBA)(Scheme 1.3).

The insertion of the oxygen is accomplished by a sequence of steps that begins by addition of the terminal oxygen of the peracid to the substrate carbonyl, followed by bond migration. The configuration of the migrating group is maintained (Turner, 1950). The concerted step involving O-O heterolysis and migration is usually rate-determining (Ogata & Sawaki, 1972). If the ketone is not symmetrical, the



Scheme 1.3 Mechanism of the Baeyer-Villiger Oxidation

structure of the product depends on which group migrates. Studies have shown that the more substituted center generally migrates (Smith, 1963). The reaction can be catalyzed by the addition of trifloroacetic acid (TFA) (Koch, 1989) and by Nickel(II) complexes (Yamada et al., 1991). Several chemical Baeyer-Villiger reactions similar to those catalyzed by our "designer yeast" (*vide infra;* Stewart et al., 1996) are shown in Table 1.1.

Applications of enantioselective Baeyer-Villiger oxidations in organic synthesis. Enantioselective oxidations are essential due to the importance of optically active oxygenated compounds as synthetic intermediates in natural product synthesis (Ojima, 1993). While the enantioselective oxidation of *mesomeric* 4-substituted cyclohexanones can be used to completely convert the starting material to product,

Table 1.1 Chemical Baeyer-Villiger Reactions

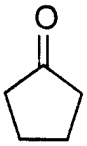
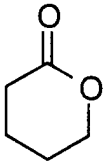
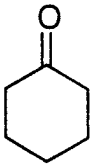
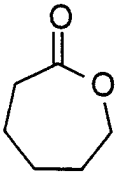
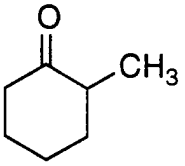
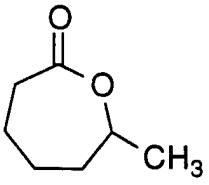
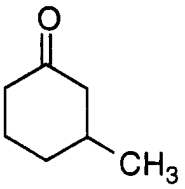
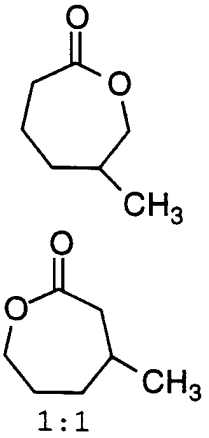
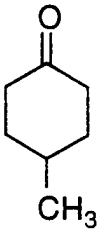
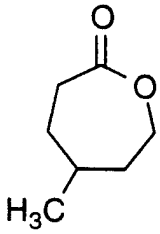
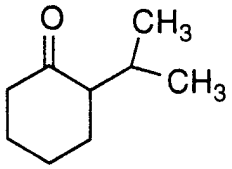
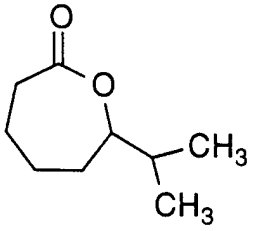
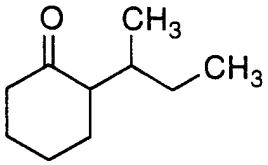
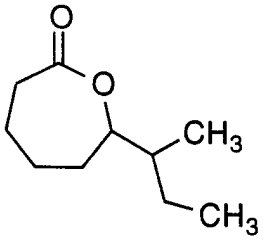
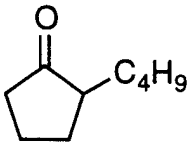
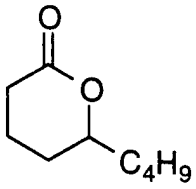
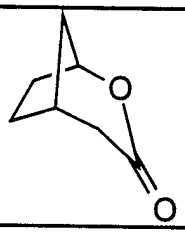
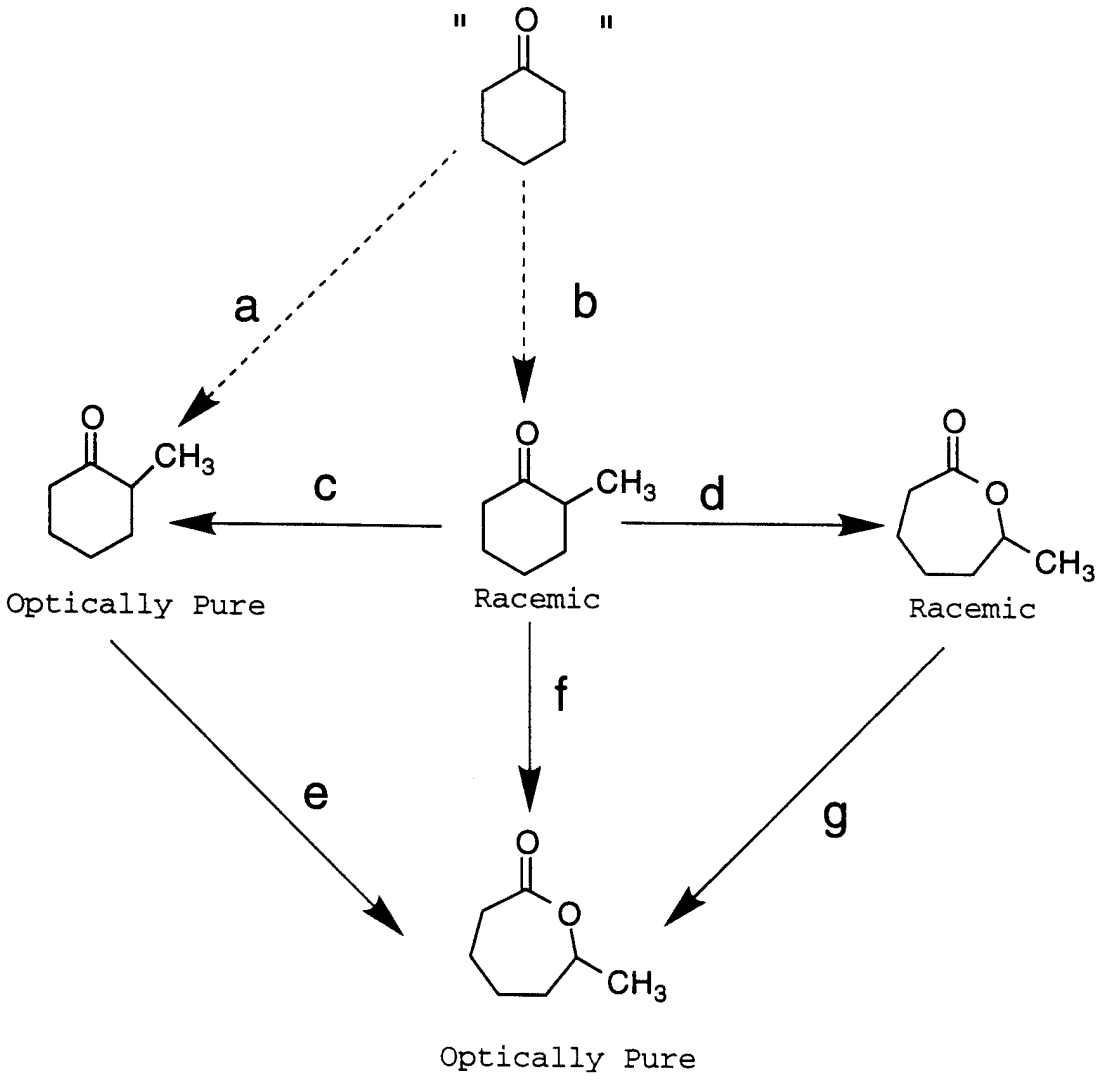
Substrate	Product	Oxidant	Yield	Reference
		CF_3CO_3H	88%	Sager (1954)
		CH_3CO_3H	85% 6.5 hrs	Starcher (1959)
		CH_3CO_3H	92% 8.5 hrs	Starcher (1959)
	 1:1	CH_3CO_3H	81% total 11 hrs	Starcher (1959)
		CH_3CO_3H	84% 9.5 hrs	Starcher (1959)

Table 1.1 continued

Substrate	Product	Oxidant	Yield	Reference
		CH_3CO_3H	85% 9 hrs	Starcher (1959)
		CH_3CO_3H	92% 13 hrs	Starcher (1959)
		H_2SO_5	ND	Parliment (1966)
		CH_3CO_3H	88%	Meinwald (1960)

Baeyer-Villiger oxidations of 2-substituted cyclohexanones are more complex. These compounds are especially valuable in synthesis as chiral building blocks. There are four principal routes to these 3-substituted caprolactones, and these are illustrated for the case of 2-methylcyclohexanone (scheme 1.4).

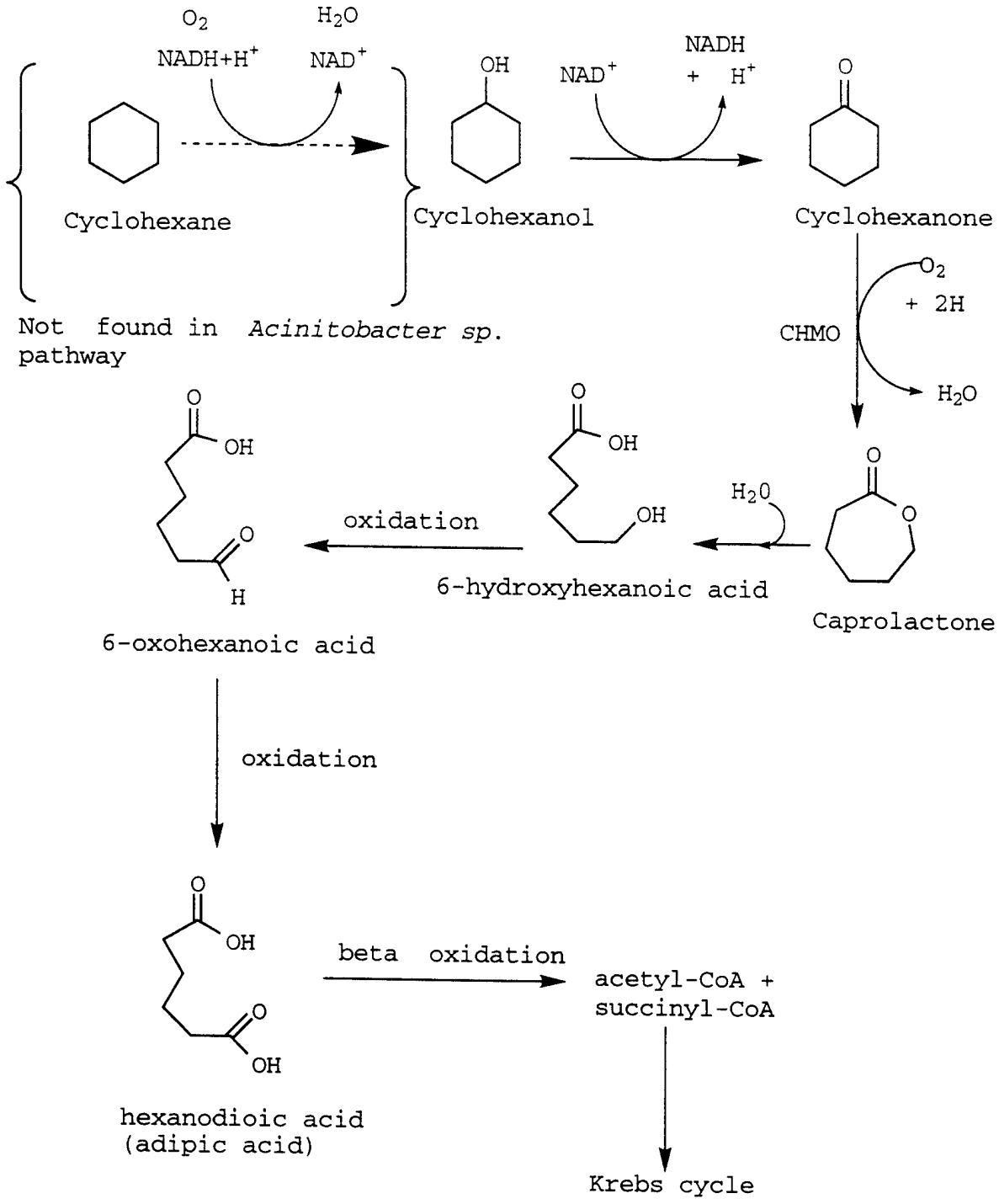


Scheme 1.4 Synthetic Pathways to Optically Active Lactones

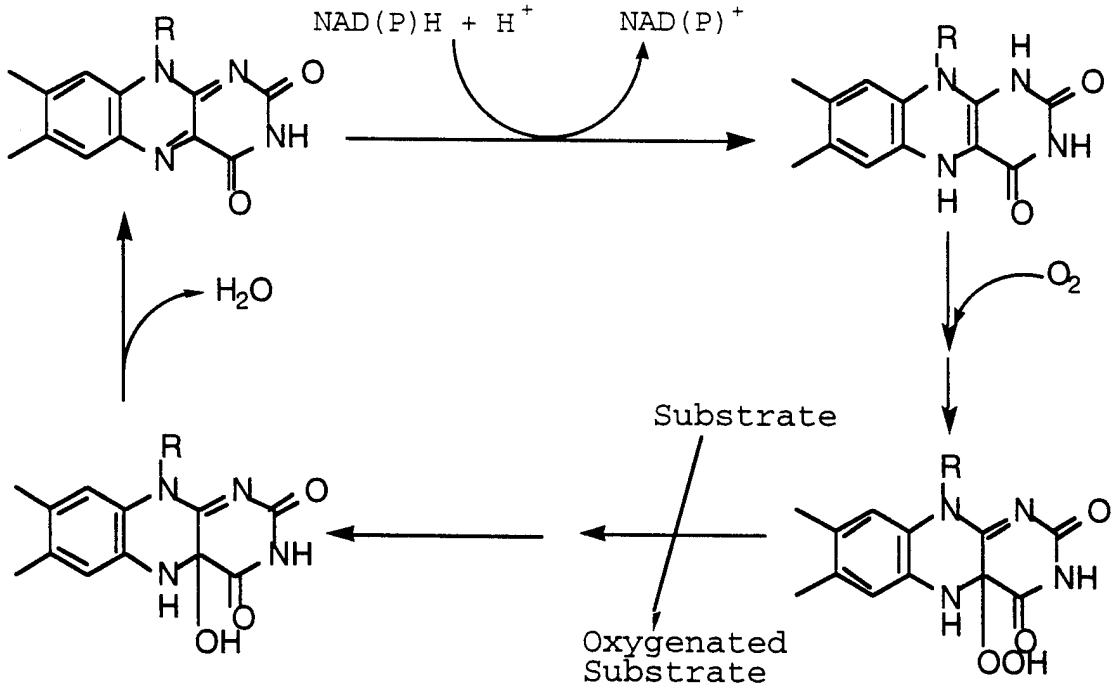
1) Starting with a prochiral or racemic starting material, one can either induce chirality or perform a kinetic resolution by a suitable catalyst (Dieter and Tokels, 1987; Katoh et al., 1994) to produce an optically active ketones (pathway a). This step can then be followed by a non-asymmetric oxidation, for example using *m*-CPBA, to give the optically active lactone (pathway e). 2) Alternatively, a racemic ketone can be produced (pathway b), and this can be followed by a non-asymmetric oxidation to afford a racemic lactone (pathway d). Finally, the lactone can be kinetically resolved to give the optically pure lactone isomers (pathway g)(Blanco et al., 1988). 3) Using an asymmetric oxidant, the racemic ketone can be kinetically resolved to give the optically active ketone (Kim et al., 1989; Matsumoto, et al., 1990)(pathway c). This step is followed by a non-asymmetric oxidation to provide a chiral lactone (pathway e).
4) Finally, the synthesis of a racemic ketone by standard synthetic methods (pathway b) can be followed by an enantioselective oxidation to a chiral lactone (pathway f). This can be accomplished by the use of a chiral catalyst (Bolm et al., 1994, 1995; Gusso et al., 1994) or the use of enantioselective enzymes (Alphand, et al., 1989; Gagnon et al., 1994, 1995; Lenn et al., 1994; Walsh and Chen, 1988). This last method is the simplist to apply and is the pathway explored in this thesis for the production of optically active lactones.

Biotransformations. The best-studied enzymatic Baeyer-Villiger reaction is catalyzed by cyclohexanone monooxygenase from *Acinitobacter sp.* (Donoghue et al., 1976). The Baeyer-Villiger reaction is catalyzed by cyclohexanone monooxygenase as part of a metabolic pathway in which cyclohexanol is converted into suitable precursors for the Krebs cycle. Using this pathway, *Acinitobacter sp.* can grow on cyclohexanol or cyclohexanone as its sole carbon source (Scheme 1.5).

Cyclohexanone monooxygenase is a member of a class of enzymes known as monooxygenases, which can be divided into four categories based on the cofactor utilized for the oxidations (Walsh and Chen, 1988). The four categories are pterin monooxygenases, copper-containing monooxygenases, cytochrome P450 oxygenases, and flavin-based monooxygenases. Cyclohexanone monooxygenase is a member of the latter family. Mechanistic studies support the formation a 4a-hydroperoxyflavin intermediate (Ryerson et al., 1982; Walsh and Chen, 1988). The flavin cofactor is oxidized during the enzymatic reaction and is returned to its active form by NADPH (Scheme 1.6). Studies have shown that cyclohexanone monooxygenase generally follows the same regioselectivity rules when dealing with 2-substituted cyclohexanones as typical Baeyer-Villiger reactions (Fouque & Rousseau, 1989). In addition, there is the possibility of some enantioselectivity in the reaction (Schwab et al., 1981, 1983).



Scheme 1.5 Cyclohexanol Metabolism in *Acinitobacter sp.*



Scheme 1.6 Catalytic Cycle (Branchaud et al., 1984)

Tables 1.2 and 1.3 contain most of the reactions known to be catalyzed by cyclohexanone monooxygenase. This list includes both whole cell reactions performed with *Acinitobacter sp.* as well as reactions involving purified enzyme. The latter examples require the addition of stoichiometric amounts of NADPH or the use of a cofactor regeneration system as outlined earlier.

Table 1.2 Biological Baeyer-Villiger Reactions Involving Asymmetric Induction.

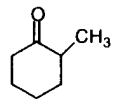
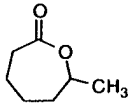
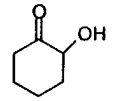
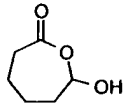
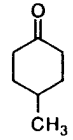
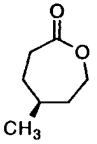
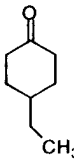
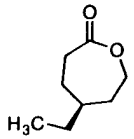
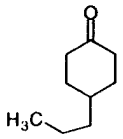
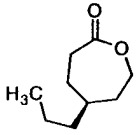
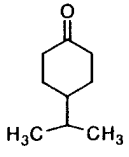
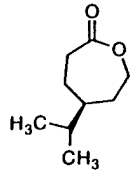
Starting Material	Product(s)	Whole cells or Purified Enzyme?	Specific Rotation	Yield	ee	Reference
Ketones:						
		Purified Enzyme	ND	ND	ND	Schwab(1983)
		ND	ND	ND	ND	Trudgill(1982)
		Whole Cells	-44.23 c1.56, $CHCl_3$	80%	> 98%	Taschner(1988)
		Purified Enzyme	-38 c5.55, $CHCl_3$	84%	>98%	Taschner(1993)
		Purified Enzyme	-38 c6.41, $CHCl_3$	80%	>98%	Taschner(1993)
		Purified Enzyme	-40 c0.44, $CHCl_3$	60%	>98%	Taschner(1993)

Table 1.2 continued

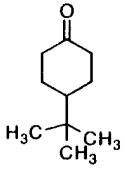
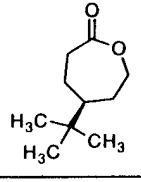
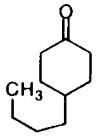
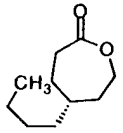
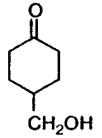
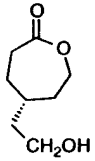
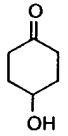
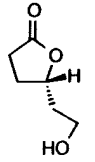
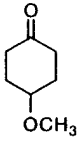
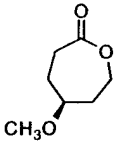
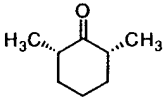
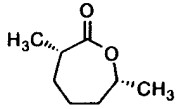
Starting Material	Product(s)	Whole cells or Purified Enzyme?	Specific Rotation	Yield	ee	Reference
		Purified Enzyme	-34.9 c0.78, $CHCl_3$	17%	>98%	Taschner(1993)
		Purified Enzyme	+18.5 c2.74, $CHCl_3$	70%	52%	Taschner(1993)
		Purified Enzyme	-6.2 c5.66, $CHCl_3$	80%	>98%	Taschner(1993)
		Purified Enzyme	-6.8 c4.75, $CHCl_3$	73%	9.6%	Taschner(1993)
		Whole Cells	-12.01 c5.56, $CHCl_3$	76%	75%	Taschner(1988)
		Whole Cells	-10.90 c1.56, $CHCl_3$	27%	> 98%	Taschner(1988)

Table 1.2 continued

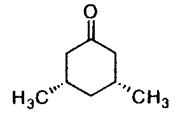
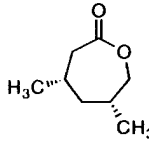
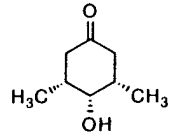
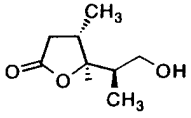
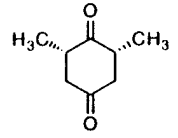
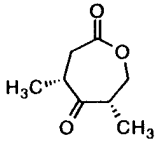
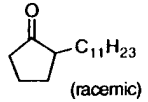
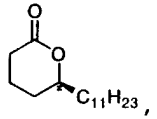
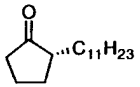
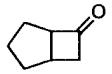
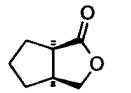
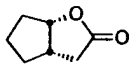
Starting Material	Product(s)	Whole cells or Purified Enzyme?	Specific Rotation	Yield	ee	Reference
		Whole Cells	-12.69 c2.45, $CHCl_3$	73%	> 98%	Taschner(1988)
		Whole Cells	-25.98 c2.54, $CHCl_3$	88%	> 98%	Taschner(1988)
		Whole Cells	-14.38 c1.62 $CHCl_3$	25%	> 98%	Taschner(1988)
 (racemic)	 , 	Whole Cells	-77.0 c1.5 Et_2O -29.8 c0.7 THF	30% 25%	95% 74%	Alphand(1990)
	 , 	Whole Cells	-93.2 c2.3, $CHCl_3$ -87.4 c1.7, $CHCl_3$	31% 36%	>95% >95%	Alphand(1992)

Table 1.2 continued

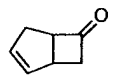
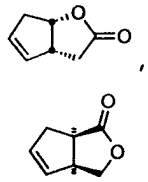
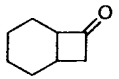
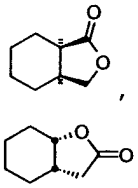
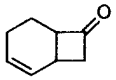
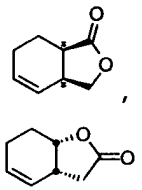
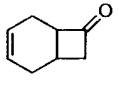
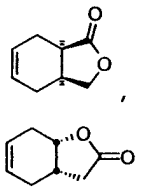
Starting Material	Product(s)	Whole cells or Purified Enzyme?	Specific Rotation	Yield	ee	Reference
		Whole Cells	-67.9 c2.3, $CHCl_3$ -102.3 c1.2, $CHCl_3$	40% 37%	> 97% >97%	Alphand(1992)
		Whole Cells	-48.2 c1.0, $CHCl_3$ -24.5 c0.9, $CHCl_3$	28% 52%	> 95% 60%	Alphand(1992)
		Whole Cells	-50.1 c0.7, $CHCl_3$ -23.7 c1.0, Et_2O	37% 43%	> 95% > 95%	Alphand(1992)
		Whole Cells	+69.7 c1.1, MeOH -49.5 c1.2, MeOH	36% 41%	> 95% > 90%	Alphand(1992)

Table 1.2 continued

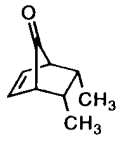
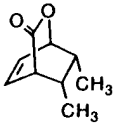
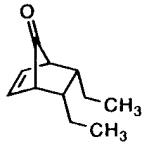
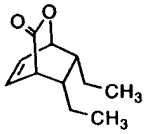
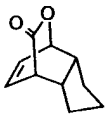
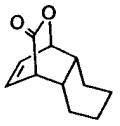
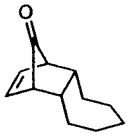
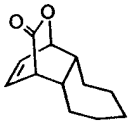
Starting Material	Product(s)	Whole cells or Purified Enzyme?	Specific Rotation	Yield	ee	Reference
		Isolated Enzyme	+59.8 c1.31, Et_2O	62%	80%	Taschner(1992)
		Isolated Enzyme	+56.8 c0.38, Et_2O	70%	> 98%	Taschner(1992)
		Isolated Enzyme	+30.0 c1.13, Et_2O	83%	93%	Taschner(1992)
		Isolated Enzyme	+40.4 c3.65, CH_2Cl_2	80%	97%	Taschner(1992)
		Isolated Enzyme	-1.60 c5.15, CH_2Cl_2	78%	> 98%	Taschner(1992)
		Isolated Enzyme	+26.0 c1.85, Et_2O	57%	> 98%	Taschner(1992)

Table 1.2 continued

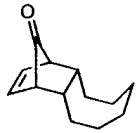
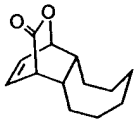
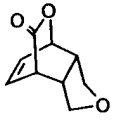
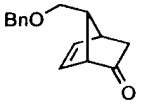
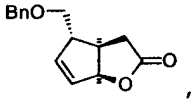
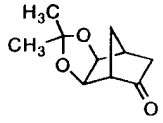
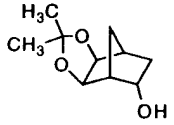
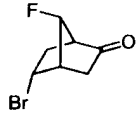
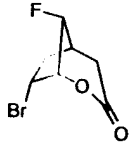
Starting Material	Product(s)	Whole cells or Purified Enzyme?	Specific Rotation	Yield	ee	Reference
		Isolated Enzyme	+16.5 c5.40, Et_2O	55%	87%	Taschner(1992)
		Isolated Enzyme	+32.0 c2.90, CH_2Cl_2	74%	> 98%	Taschner(1992)
	 	Whole Cells	+189.7 c2.04, $CHCl_3$ *endo*, +60.8 c2.32, $CHCl_3$ *exo*, 3.0 c0.53, $CHCl_3$	26% 35%	85% 95%	Königsberger(1991)
		Whole Cells	-2.0 c2.43, $CHCl_3$	64%	34%	Königsberger(1991)
		Whole Cells	N.D.	35%	> 95%	Levitt(1990)

Table 1.2 continued

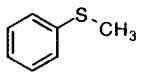
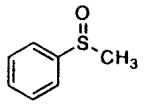
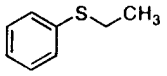
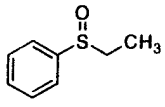
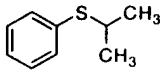
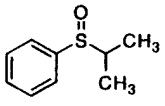
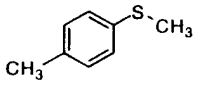
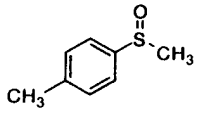
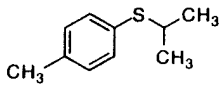
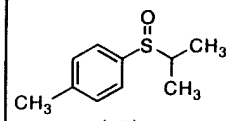
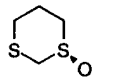
Starting Material	Product(s)	Whole cells or Purified Enzyme?	Specific Rotation	Yield	ee	Reference
Sulfides:						
	 (*R*)	Whole Cells	N.D.	97%	85%	Beecher(1994)
	 (*R*)	Whole Cells	N.D.	57%	27%	Beecher(1994)
	 (*R*)	Whole Cells	N.D.	43%	29%	Beecher(1994)
	 (*S*)	Whole Cells	N.D.	94%	26%	Beecher(1994)
	 (*S*)	Whole Cells	N.D.	58%	47%	Beecher(1994)
		Purified Enzyme	N.D.	81%	>98%	Colonna(1995)

Table 1.2 continued

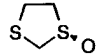
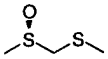
Starting Material	Product(s)	Whole cells or Purified Enzyme?	Specific Rotation	Yield	ee	Reference
		Purified Enzyme	N.D.	94%	>98%	Colonna(1995)
		Purified Enzyme	N.D.	92%	>98%	Colonna(1995)

Table 1.3 Biological Baeyer-Villiger Reactions Not Involving Asymetric Induction.

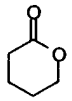
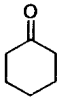
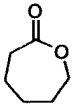
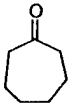
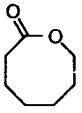
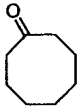
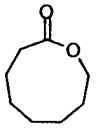
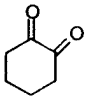
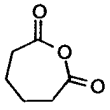
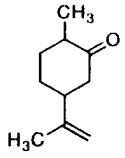
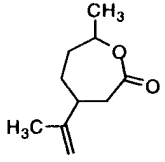
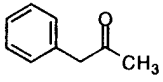
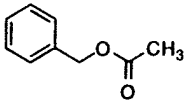
Starting Material	Product(s)	Whole Cells or Purified Enzyme?	Yield	Reference	Notes
Ketones:					
		Purified Enzyme	N.D.	Trudgill(1982)	
		Purified Enzyme	N.D.	Donoghue(1976)	
		Purified Enzyme	N.D.	Trudgill(1982)	
		Purified Enzyme	N.D.	Trudgill(1982)	
		Purified Enzyme	N.D.	Trudgill(1982)	
		Purified Enzyme	N.D.	Abril(1989)	Reaction proceeded for 10 days.
		Purified Enzyme	N.D.	Branchaud(1985)	*V*max was 7% of the cyclohexanone value.

Table 1.3 continued.

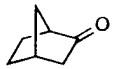
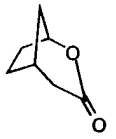
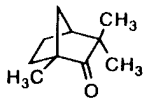
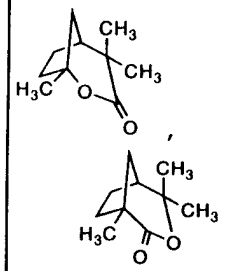
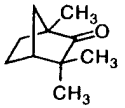
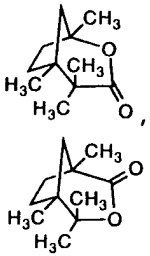
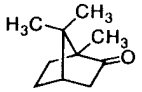
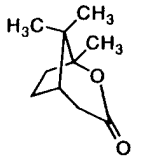
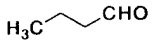
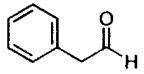
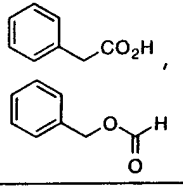
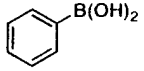
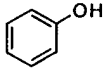
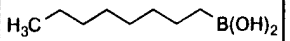
Starting Material	Product(s)	Whole Cells or Purified Enzyme?	Yield	Reference	Notes
		Purified Enzyme	81%	Abril(1989)	Reaction proceeded for 5 days.
		Purified Enzyme	76% overall	Abril(1989)	Reaction proceeded for 8 days.
		Purified Enzyme	79% overall	Abril(1989)	Reaction proceeded for 10 days.
		Purified Enzyme	89%	Abril(1989)	Reaction proceeded for 10 days.

Table 1.3 continued

Starting Material	Product(s)	Whole Cells or Purified Enzyme?	Yield	Reference	Notes
Aldehydes:					
		Purified Enzyme	N.D.	Branchaud(1985)	*V*max was 35% of the cyclohexanone value.
		Purified Enzyme	N.D.	Branchaud(1985)	*V*max was 15% of the cyclohexanone value.
Sulfides:					
		Purified Enzyme	N.D.	Branchaud(1985)	*V*max was 87% of the cyclohexanone value.
Boronic Acids:					
		Purified Enzyme	N.D.	Branchaud(1985)	*V*max was 10% of the cyclohexanone value.
		Purified Enzyme	N.D.	Branchaud(1985)	*V*max was 145% of the cyclohexanone value.

1.4 Solubility and Toxicity of Substrates

General. The use of enzymes and whole cell systems for the bioconversion of organic substrates is often fraught with problems of substrate solubility. In addition, whole cell systems can be subject to toxicity effects caused by the substrates. There are several ways, including the use of using organic solvents and surfactants, to mitigate the toxicity effects and increase substrate solubility. Unfortunately, when living cells are used as the biocatalyst, the presence of organic solvents or surfactants will often result in inhibitory or toxic effects (Nakamatsu et al., 1983; Freeman et al., 1987).

Surfactants. A procedure commonly used in industry involves the addition of surfactants such as Triton, Tween 80 and Span to increase substrate solubility (Nakamatsu et al., 1983). These reagents are inexpensive and provide some increase in substrate solubility and increases in reaction rates. While other systems have been shown to be better, they are significantly more expensive.

Cyclodextins. Cyclodextrins are cyclic oligosaccharides derived from starch. The most common cyclodextrins are made of 6, 7, and 8 glucose units linked in a circular array. These donut-shaped molecules form a hydrophobic cavity on the inside surrounded by a hydrophilic shell (Figure 1.1).

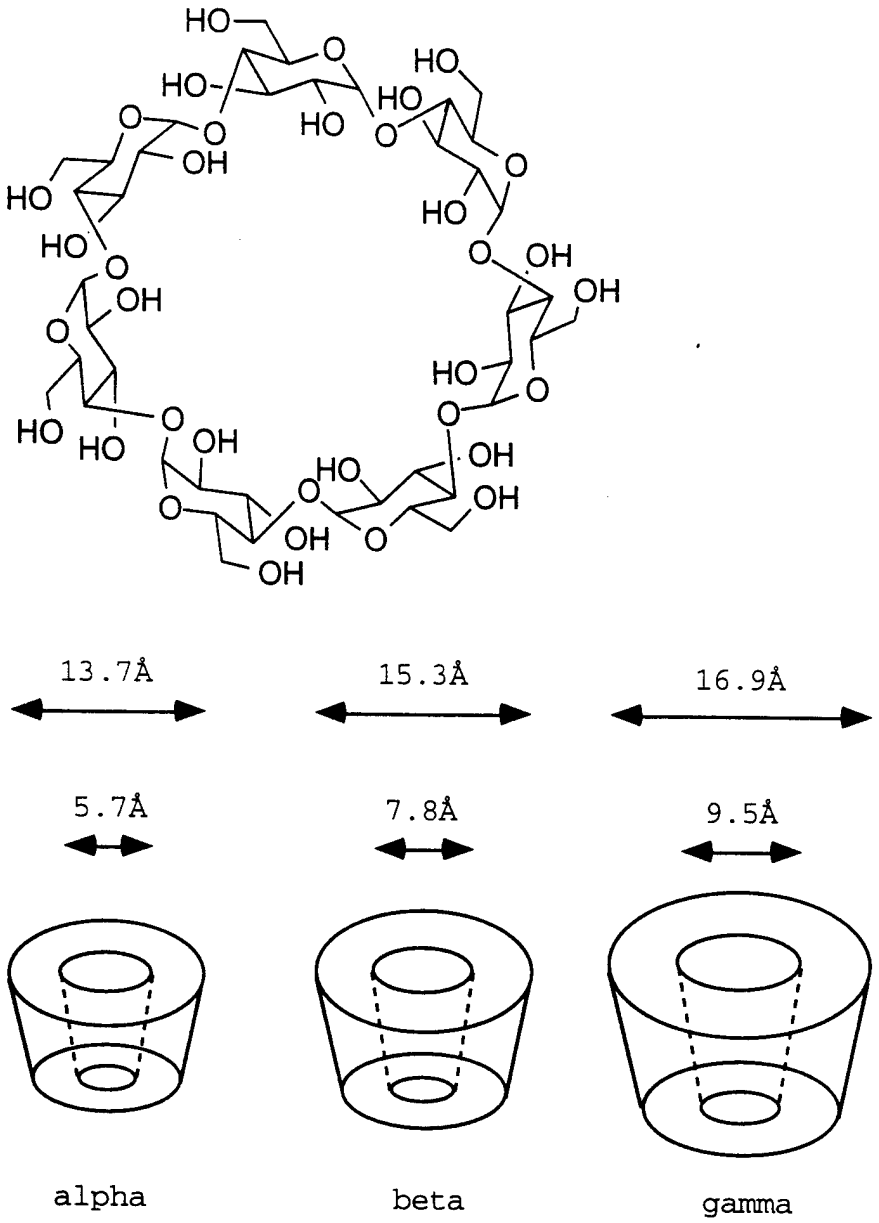
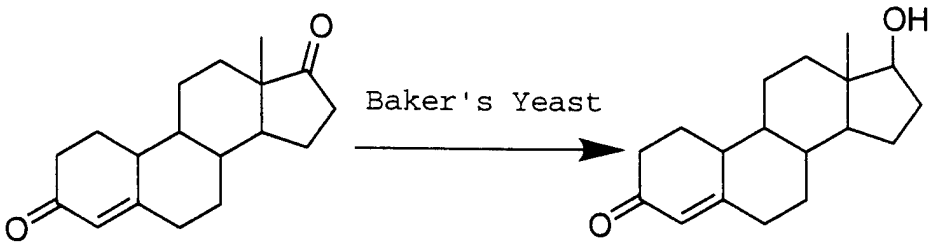


Figure 1.1 Cyclodextrins

This hydrophobic cavity can accommodate a suitably sized guest or portion of the guest molecule. Cyclodextrins have been used by several researchers to increase the solubility of organic substrates in aqueous media and to mitigate the toxic effects that many organics have on whole cell systems (Bar et al., 1989; Goetschel and Bar, 1991; Hesselink et

al., 1989; Jadoun et al., 1993a, 1993b; Singer et al., 1991). A great deal of work has been done using *Saccharomyces cerevisiae* to reduce highly hydrophobic compounds such as androstenedione aided with presence of cyclodextrins (Singer et al., 1991)(Scheme 1.7).



Androstenedione Testosterone

Scheme 1.7 Androstenedione reduction

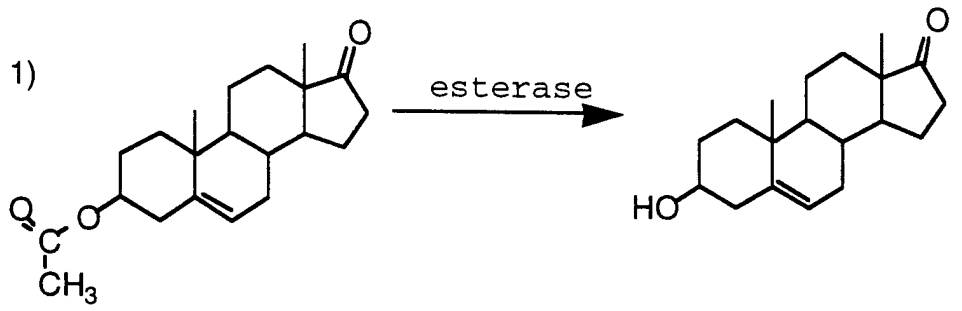
Cyclodextrins offer two benefits to whole cell biotransformations. First, they have been shown to mitigate the toxic effects of organics on living cells, probably by decreasing the amount of free organics in solution to a level that can be tolerated by the cell (Bar et al., 1989a). Secondly, they have been shown to dramatically increase the solubility of many hydrophobic and lipophilic compounds (Bar et al., 1989a, 1989b; Hesselink et al., 1989; Jadoun et al., 1993a, 1993b; Singer et al., 1991). An example of the dramatic effect of the addition of cyclodextrins is shown by cholesterol, which has a solubility of 1.8 mg/L in water (Bar et al., 1989a). However, when methylated β-cyclodextrin is added to 50 mM, the solubility of cholesterol increased to 4 g/L (Jadoun et al., 1993).

Liposomes. Diacyl phospholipids spontaneously form structures called liposomes when placed in an aqueous media. Liposomes are lipid bilayers with diameter greater than 500 nm (Goetschel et al., 1992; Gregoriadis et al., 1984). When these are present in aqueous media, the solubility of organic compounds can be enhanced dramatically. Using liposome preparations, the solubility of cholesterol can be increased to 20 g/L or more (Collins et al., 1982). The structure of liposomes is similar to that of the membranes of the cells. The organics partition into the micelles and not into the cell membranes, which reduces toxicity.

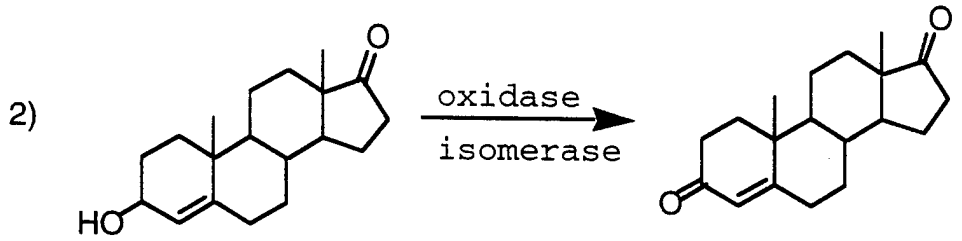
One-phase systems. One-phase systems use water miscible co-solvents such as ethanol and ethylene glycol to increase the solubility of organic substrates (Beecher et al., 1995). For example, ethylene glycol at 30%(v/v) in water will increase the solubility of hydrocortisone by 10-fold (Freeman et al., 1987). Unfortunaately, this increased solubility is accompanied by decreased reaction rates due to a decrease in the solubility of oxygen.

Two-phase systems. In two-phase systems, an organic solvent such as octane is used to decrease the substrate concentration in the aqueous phase. Equal volumes of aqueous media containing the biocatalytic cells and octane are mixed vigorously. Under these conditions, substrate solubility can be enhanced significantly and also increase reaction efficiency (Boeren and Laane, 1986). Scheme 1.8 shows some

examples of whole cell transformations completed under these conditions.



androstenolone-acetate dehydro-epiandrosterone



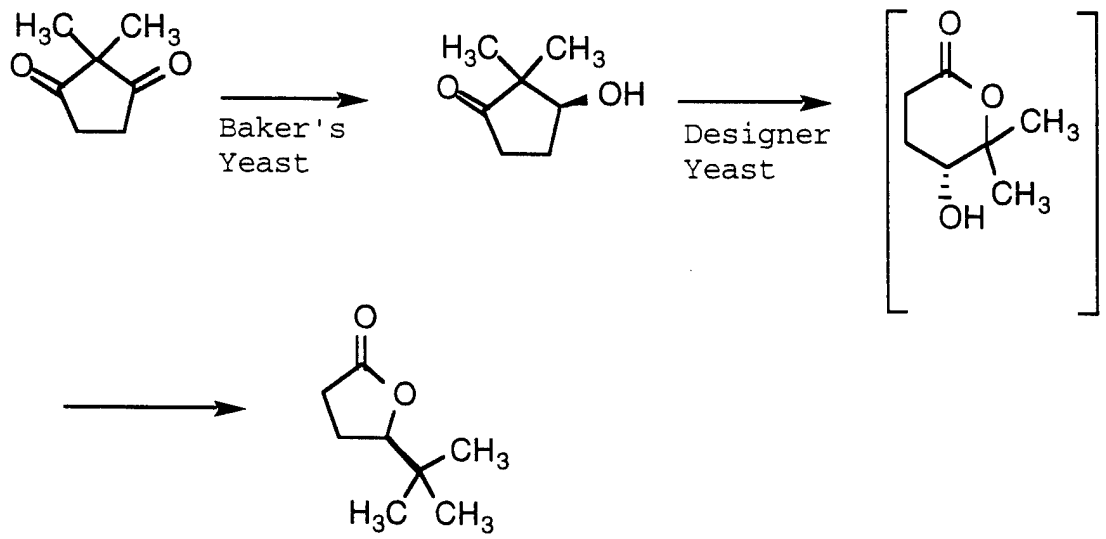
dehydro-epaindrosterone 4-androstene-3,17 -dione

Scheme 1.8 (Boeren et al., 1987)

1.5 Long Term Goals

The long term goal of this research is to produce various yeast strains that express synthetically useful enzymes individually or that co-express several enzymes to create a novel biochemical pathway within yeast. Our goal is to create strains that catalyze a whole class of reactions, instead of the current methods which tend to catalyze very specific reactions. In addition, the natural tendency of yeast to reduce chemical compounds can be coupled with these

expression systems. Scheme 1.9 is an example of a possible engineered pathway.



Scheme 1.9 Possible Engineered Pathway

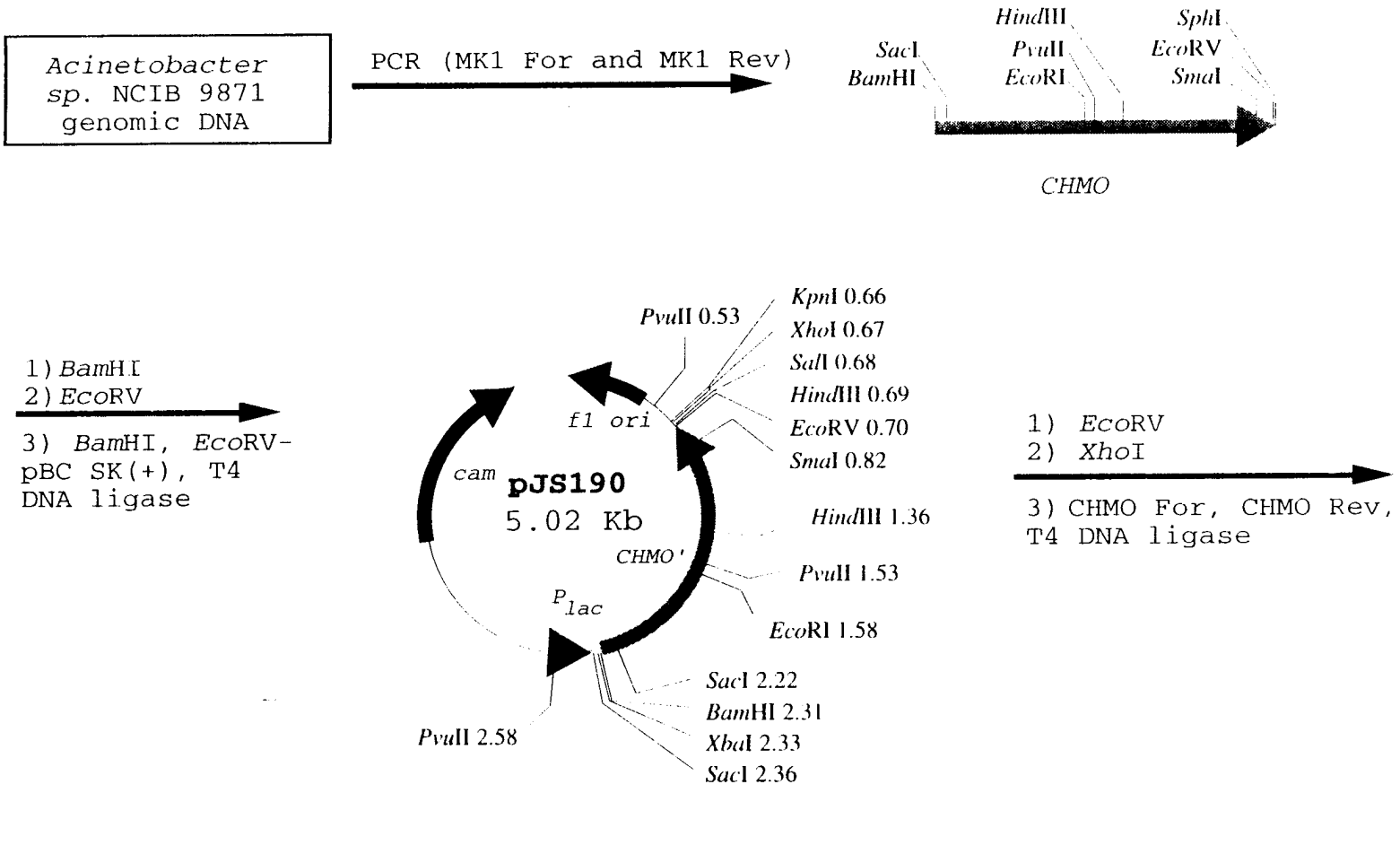
In this case, a reduction catalyzed by native yeast enzymes (Lanzilotta et al., 1975) is followed by a Baeyer-Villiger oxidation mediated by the designer yeast. The six membered lactone would then spontaneously rearrange to the final product. Finally, these designer yeast could be dried, as is baker's yeast, and packaged as an off-the-shelf chiral reagent for synthetic chemists. The first step in reaching this goal was the construction of a plasmid containing cyclohexanone monooxygenase for expression in baker's yeast.

CHAPTER 2
RESULTS

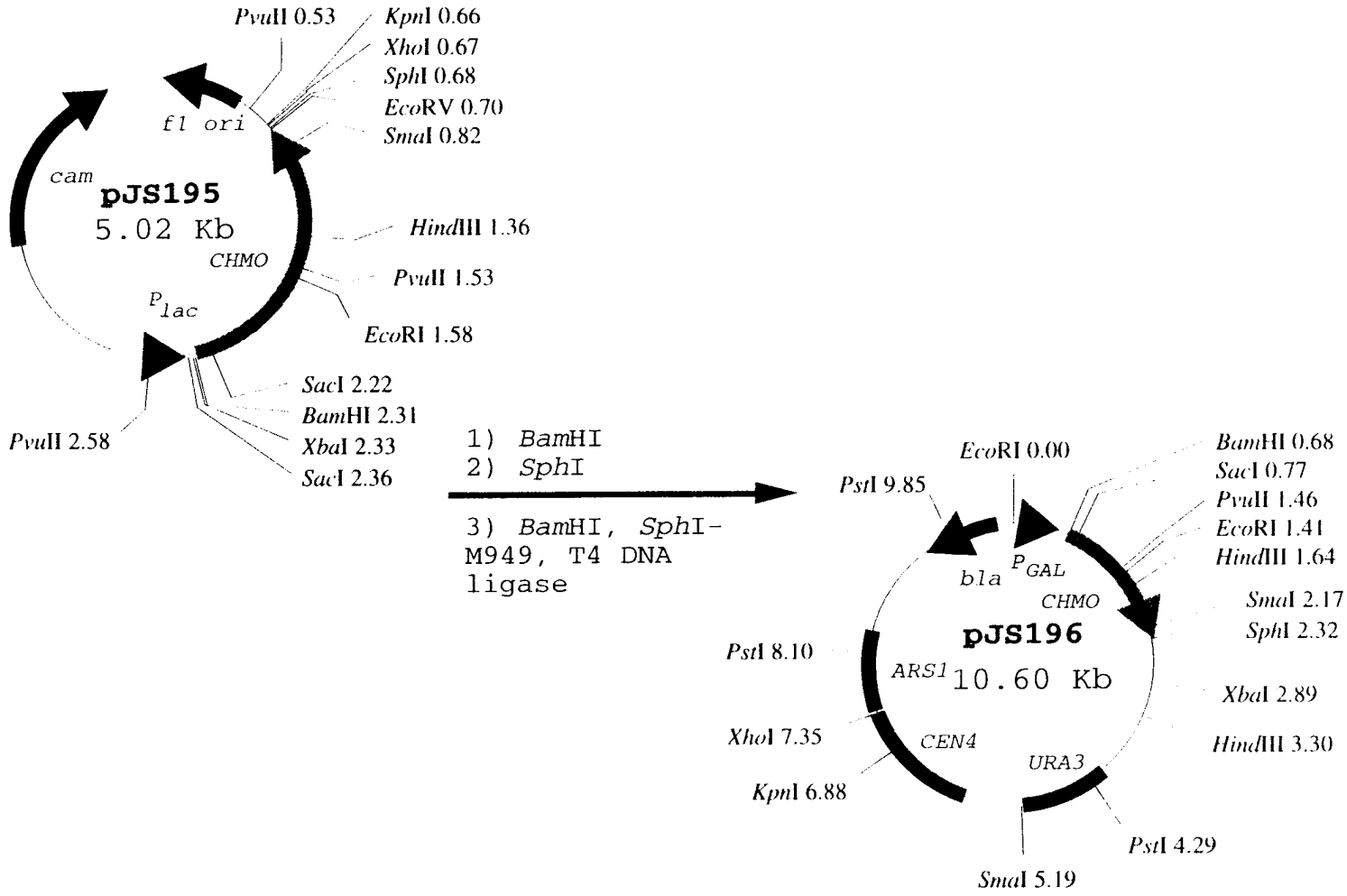
2.1 Construction of Expression Plasmids

Construction of a first-generation expression plasmid. Several plasmids were constructed to express cyclohexanone monooxygenase in baker's yeast. The cyclohexanone monooxygenase gene (*CHMO*) was amplified from *Acinetobacter sp.* genomic DNA using two PCR primers (MK1 For and MK1 Rev) designed using the known sequence (Chen et al., 1988)(Scheme 2.1). These primers incorporated suitable restriction sites flanking the structural gene for cyclohexanone monooxygenase (*CHMO*). The resulting double-stranded DNA was then cloned into pBC SK(+) as a *Bam*HI, *Eco*RV cassette giving pJS190. This cloning strategy deleted the DNA encoding the C-terminal 5 amino acids. The 3′ end of the *CHMO* gene was then added by digesting pJS190 with *Eco*RV and *Xho*I, then inserting a double-stranded DNA segment between these sites (formed from CHMO For and CHMO Rev) to afford plasmid pJS195. The initial yeast expression plasmid, pJS196 (figure 2.1), was created by subcloning the *CHMO* gene as a *Bam*HI, *Sph*I cassette between these sites in plasmid M949 (Bruce Stillman, University of Utah) to produce pJS196.

Scheme 2.1 pJS196 Construction



Scheme 2.1 continued



The 5′ end of the *CHMO* gene in pJS196 was sequenced, and three base pairs were missing within the first 6 codons at the N-terminus. However, since the gene product was enzymatically active, (*vide infra*) and the three deletions preserved the reading frame, these errors were not corrected.

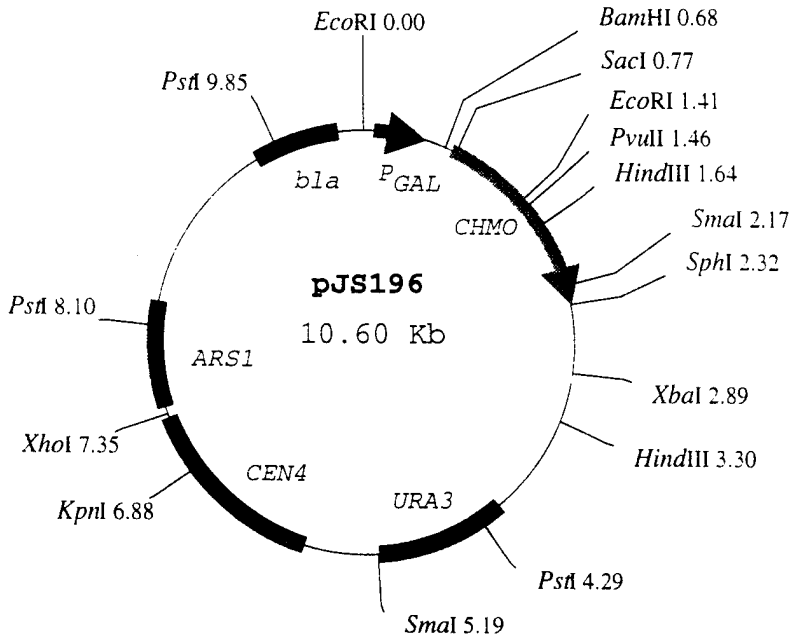


Figure 2.1 pJS196 Yeast Expression Plasmid

In pJS196, expression of the *CHMO* gene is controlled by the *GAL* promoter. The *ARS-CEN* yeast origin of replication maintains the plasmid copy number at 1-2 copies per cell. The *URA3* gene allows selection in uracil-minus media. This plasmid also contains the ampicillin resistance gene (*bla*) and the *col*E1 origin for maintenance and cloning in *E. coli*.

Construction of a second-generation expression plasmid. Since pJS196 was designed for low expression levels in yeast,

two other expression plasmids were constructed that were designed for progressively higher levels of expression. The first of these two plasmids was based on the Invitrogen plasmid pYES2. Scheme 2.2 shows the construction strategy used. The *CHMO* gene was excised from pJS196 as a *Bam*HI, *Sph*I fragment and cloned between the same sites in pYES2, affording pKR001. The resulting plasmid, pKR001 (figure 2.2), contained the following major change from pJS196. The *ARS-CEN* yeast origin of replication was replaced by the 2-micron circle yeast origin of replication, which should result in copy numbers per cell of 10 to 12. All other major characteristics of the expression system remained the same. It is this plasmid that was used for most of the work done in this thesis.

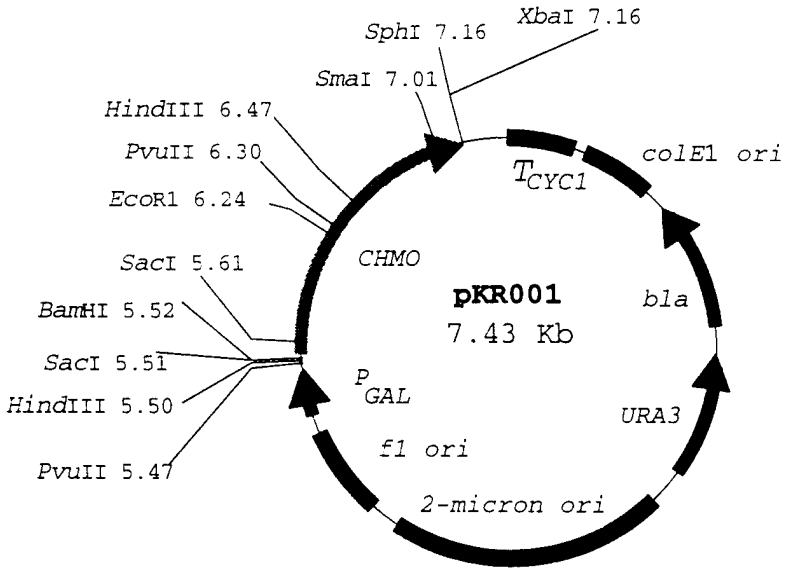
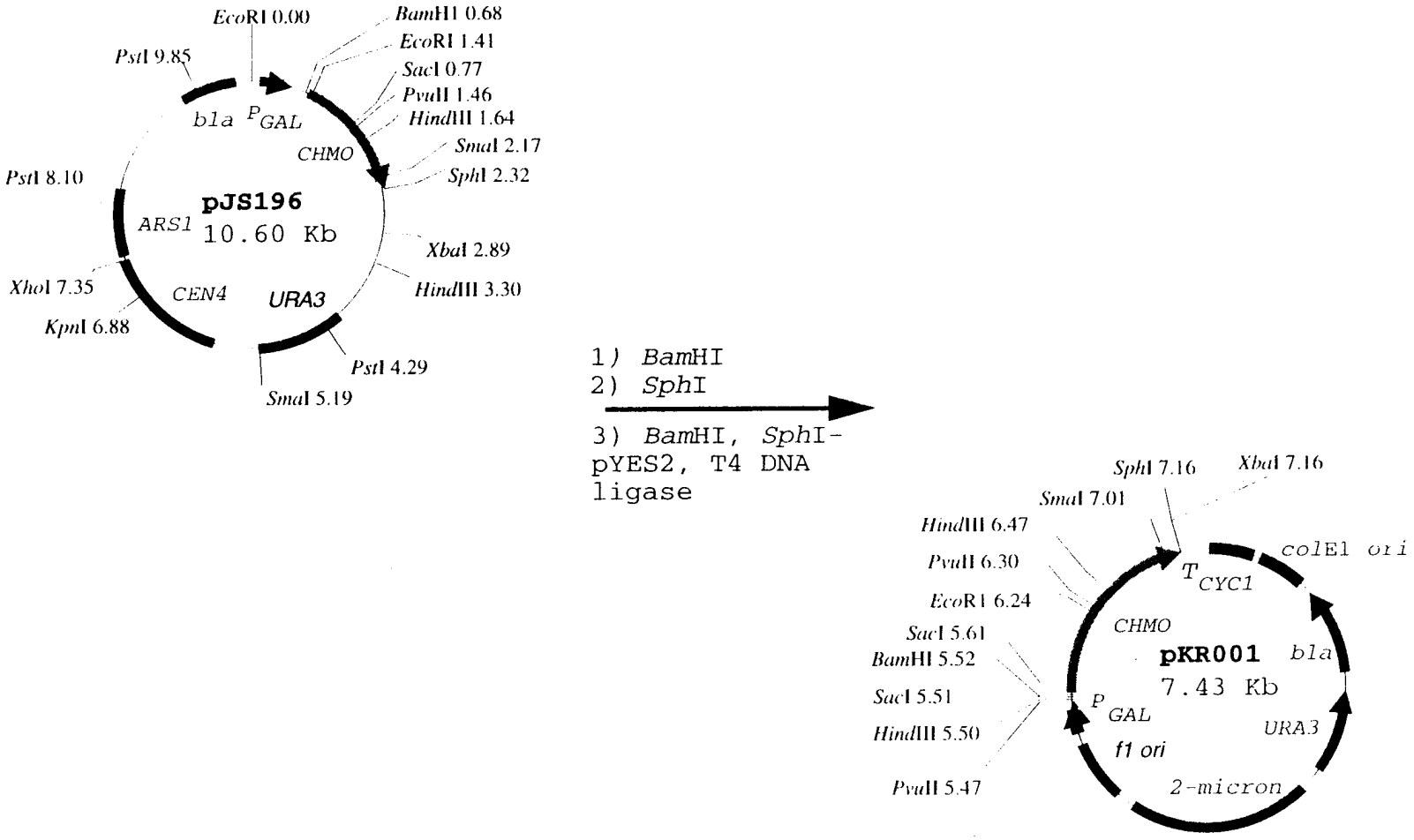


Figure 2.2 pKR001 Yeast Expression Plasmid

Scheme 2.2 pKR001 Construction



Construction of a third-Generation expression plasmid.

The next plasmid constructed was a constitutive expression plasmid containing the cyclohexanone monooxygenase gene based on plasmid pG-3, a generous gift of Keith Yamamoto (Schena et al., 1991)(Figure 2.3).

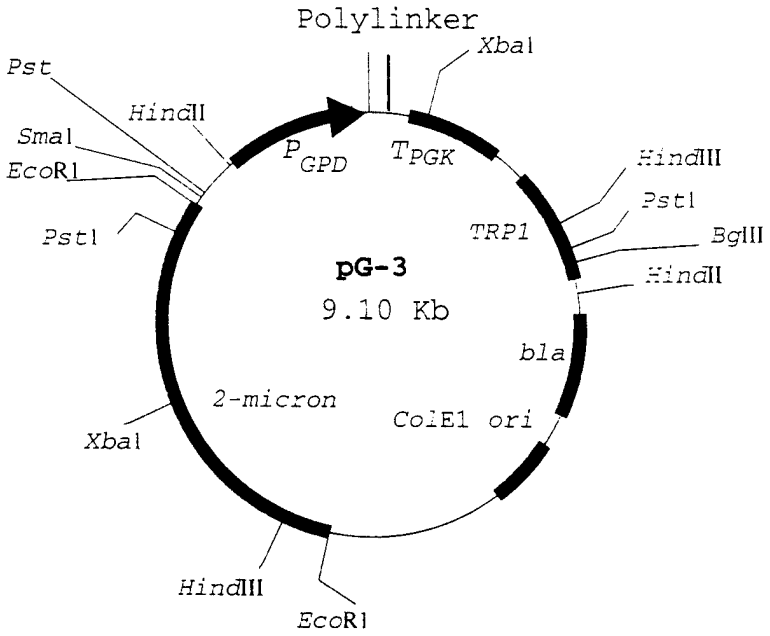
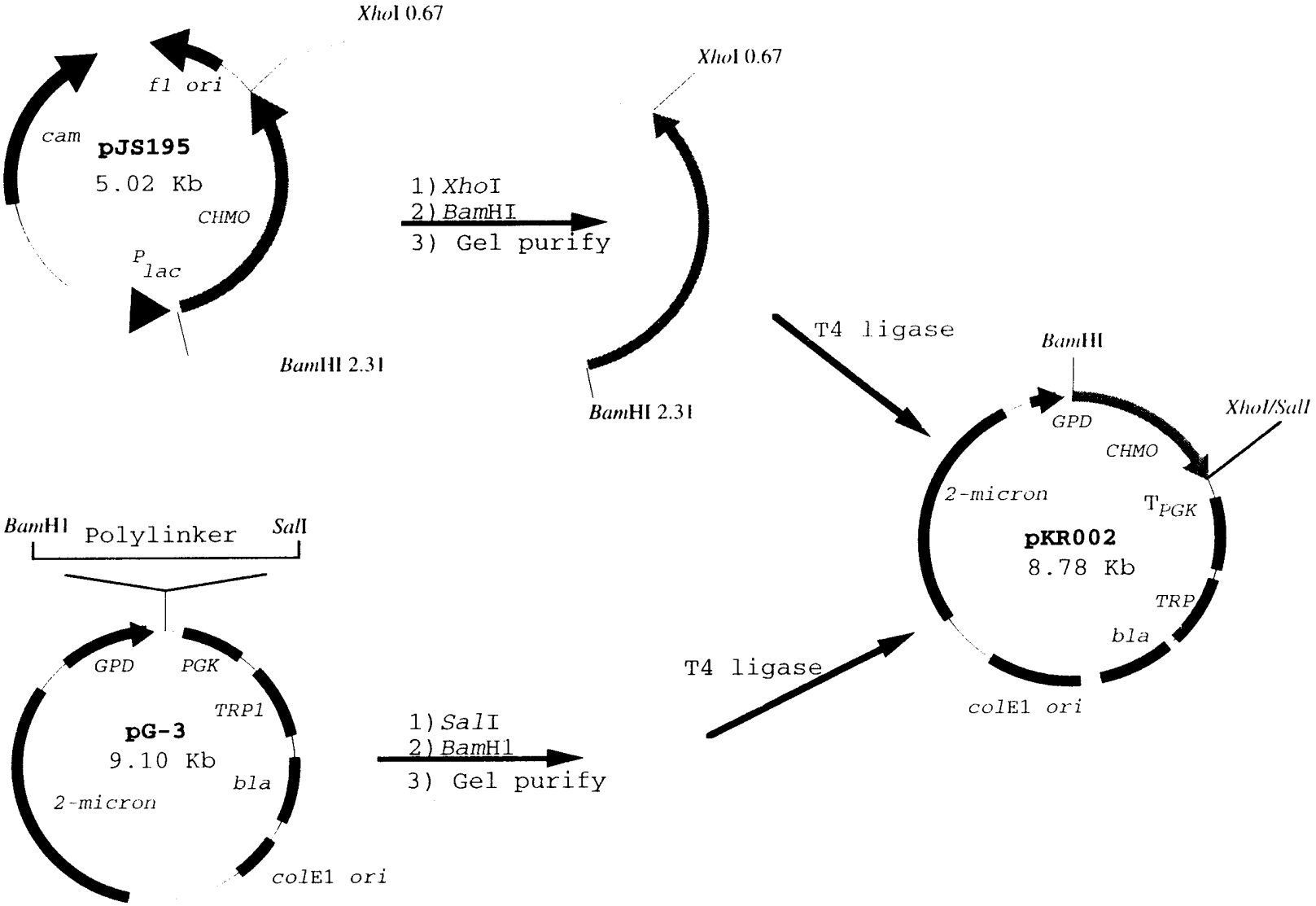


Figure 2.3 Constitutive Expression Plasmid pG-3

The cyclohexanone monooxygenase gene was cloned into this plasmid following the method outlined in scheme 2.3. The *CHMO* gene was excised from pKR001 by digesting with *Bam*HI and *Xho*I, then this fragment was subcloned between the *Bam*HI and *Sal*I sites of pG-3. In plasmid pKR002 (figure 2.4) the *CHMO* gene was under control of the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter. This is a constitutive promoter with very high levels of expression. The 2 micron circle origin of replication was designed to maintain a copy

Scheme 2.3 pKR002 Construction



number of 10-12 in yeast cells. This plasmid uses the *TRP1* gene (rather than *URA3*) for selection using an auxotrophic yeast grown in media that lacks tryptophan.

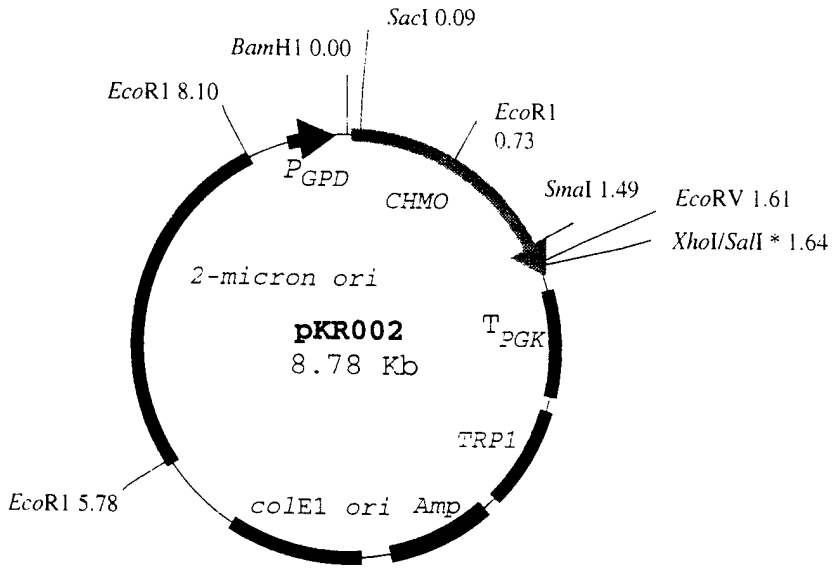


Figure 2.4 pKR002 Constitutive Yeast Expression Plasmid

Plasmids pJS196, pKR001 and pKR002 provide a series of expression vectors with progressively higher levels of gene expression. Plasmid pJS196, at 1-2 copies per cell, and controlled by the *GAL* promoter gives us the lowest expression. This may be useful when dealing with enzymes that are toxic to the yeast cells or whose reaction products are toxic to the cells. Plasmid pKR001 is an intermediate-level expression system. Copy numbers per cell are expected to range from 10-12, but once again the cyclohexanone monooxygenase gene is under the control of the *GAL* promoter. Theoretically, pKR002 will give us our highest levels of gene

expression, but we cannot control the expression. In addition, due to the metabolic drain exerted on the yeast by this plasmid, it proved much more difficult to maintain high plasmid retention rates.

Construction of an *E. coli* overexpression plasmid. An *E. coli* overexpression plasmid was constructed using the T7 expression system. The construction followed the method outlined in scheme 2.4 and resulted in plasmid pKR004 (figure 2.5). The major change required was the presence of a ribosome binding site (RBS) in front of the cyclohexanone monooxygenase gene to allow for expression in prokaryotic systems. This plasmid contains the cyclohexanone monooxygenase gene under the control of the T7 promoter and allowed us to overexpress cyclohexanone monooxygenase in the *E. coli* strain Bl21[DE3]. Preliminary studies indicated that this system did not efficiently overexpress the enzyme.

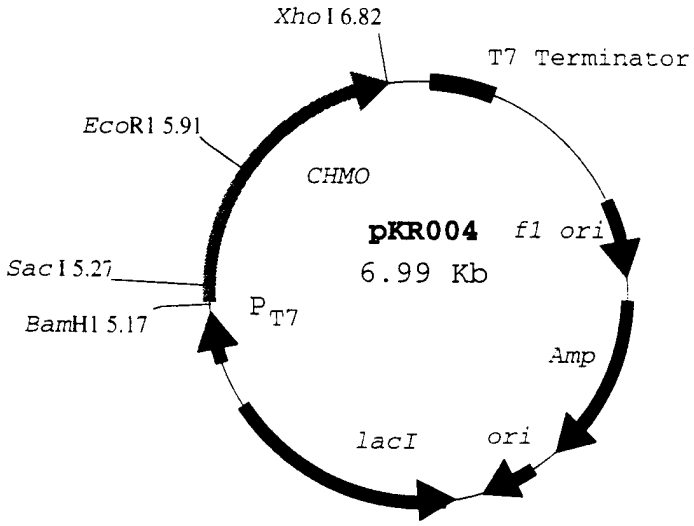
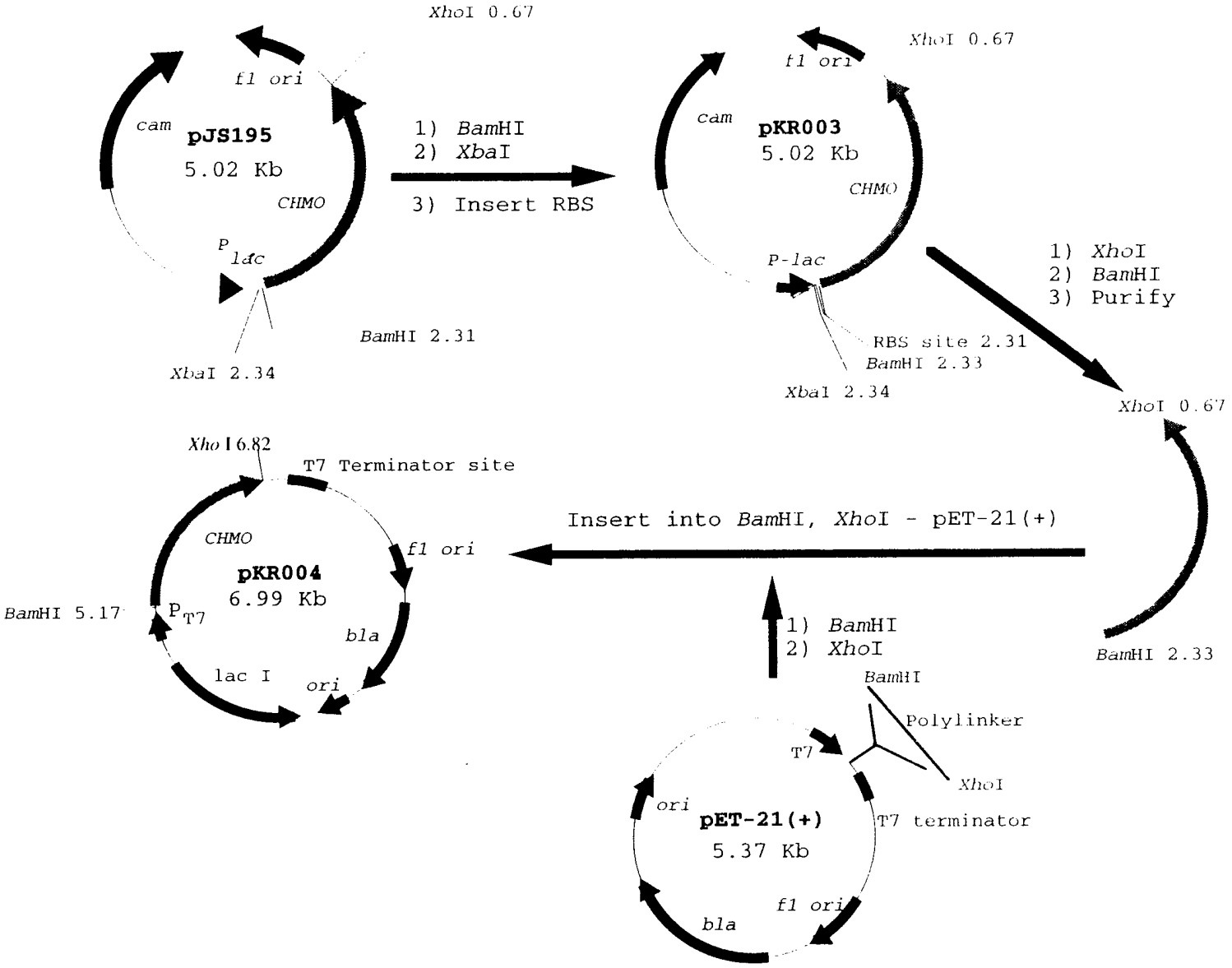


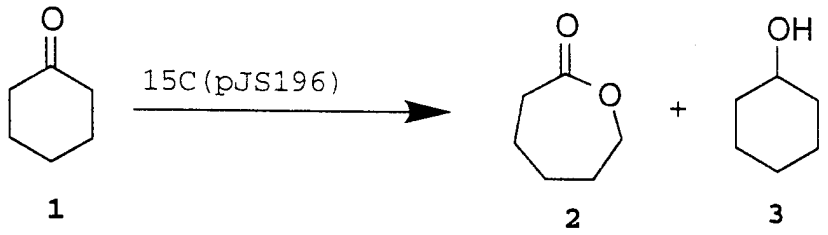
Figure 2.5 *E. coli* Expression Plasmid pKR004

Scheme 2.4 pKR004 Construction



2.2 Standard Growth Conditions

General. A series of experiments was conducted to determine the optimal conditions for oxidation of cyclohexanone using the transformed yeast strain, 15C(pJS196). We were guided in these initial studies by the conditions normally used for performing enantioselective reductions using baker's yeast. Our aim was to maximize the conversion of cyclohexanone **1** to caprolactone **2** and minimize the yeast-mediated reduction to cyclohexanol **3** (scheme 2.5).

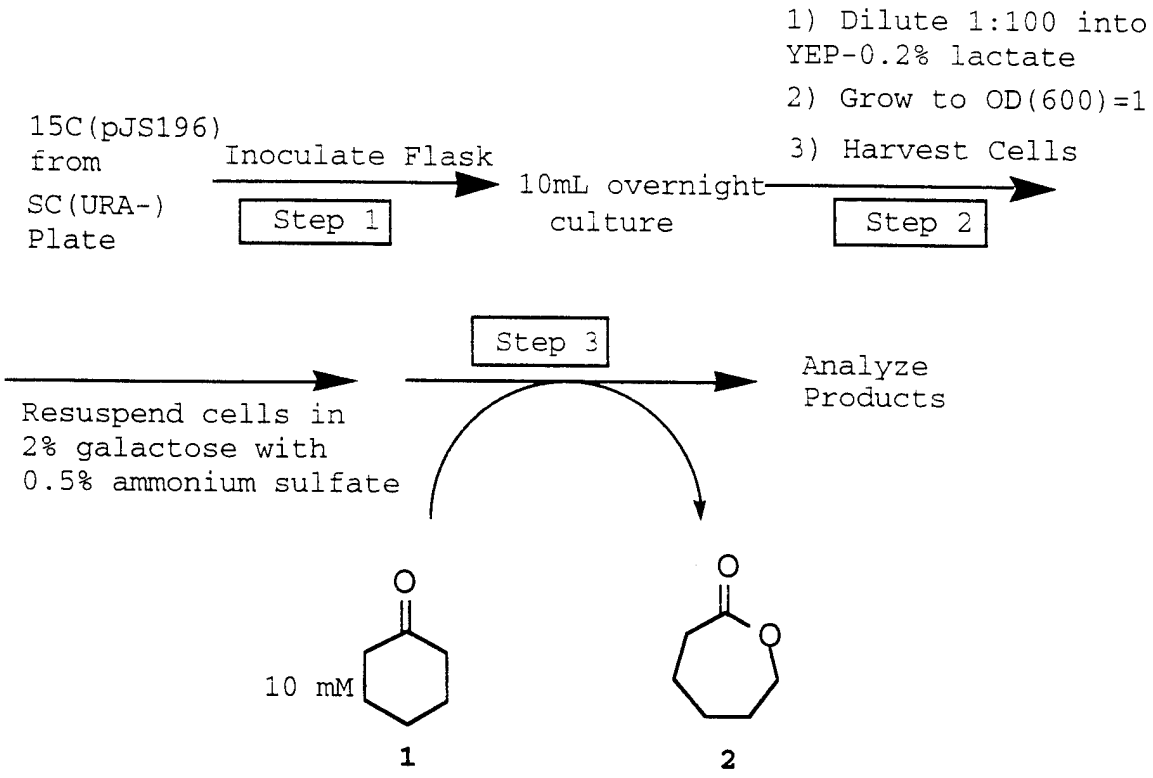


Scheme 2.5 Cyclohexanone Biotransformation

Growth on lactate. Because the *GAL* promoter is only active in the absence of glucose, we initially tried to grow our yeast in a citrate-buffered medium (pH 7.5) with lactic acid as the carbon source (scheme 2.6). These conditions were selected since they are commoly used for protein overexpression in yeast (Guthrie & Fink, 1991). The growth media for steps 1 and 2 was YEP-0.2% lactic acid while that for step 3 was water containing 0.5% ammonium sulfate.

Biotransformations under these conditions typically resulted in less than 10% caprolactone formed after 24 hours,

according to NMR analysis of the crude product. Given these results and the poor cell growth observed, we explored other carbon sources for the biotransformation.



Scheme 2.6 Initial Growth Strategy

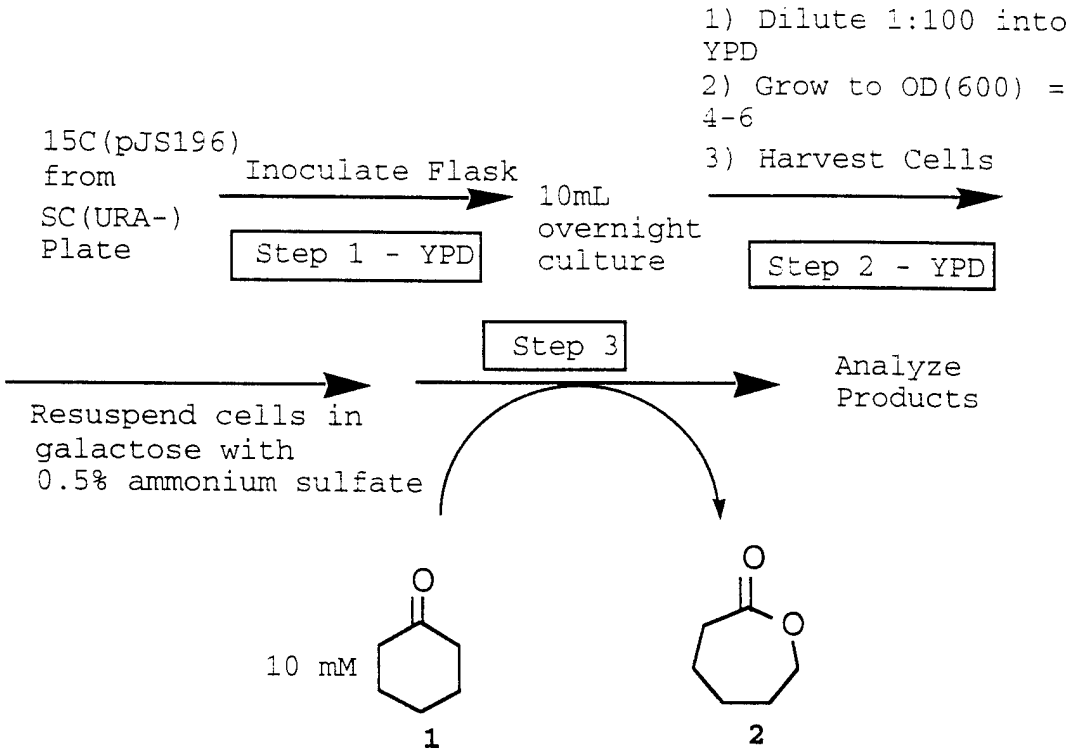
Carbon source determination. A series of experiments was conducted in order to determine the best carbon source for growing 15C(pJS196) in liquid culture, starting from a single colony picked from the storage plate (step 1 in scheme 2.6). The yeast cells were grown from the storage plate in each of the carbon sources listed in table 2.1, and their growth was monitored by absorbence at 600nm. Based on the results (table 2.1), we chose glucose as our carbon source.

Table 2.1 Carbon Source Determination

Carbon Source	OD(600) after 46 hrs
Glucose	15
Fructose	5.5
Raffinose	3.8
Lactic Acid	0.18

When using glucose for growing the engineered yeast, it is important that all of the glucose be removed from the media prior to inducing enzyme production with galactose due to the inhibitory effect of the glucose on the galactose promoter. Thus, we developed a two-step procedure in which cells were initially grown in glucose, harvested, washed, and then used for biotransformations in the presence of galactose. This washing was accomplished by centrifuging the cells at 3000g and re-suspending the cell pellet in TE, pH 7.5. This was done 3 times prior to inoculating the reaction flask with the cells.

Optimizing galactose concentration. Once the conditions for growing and harvesting the engineered yeast cells had been established, we varied the concentration of galactose used to induce production of cyclohexanone monooxygenase.



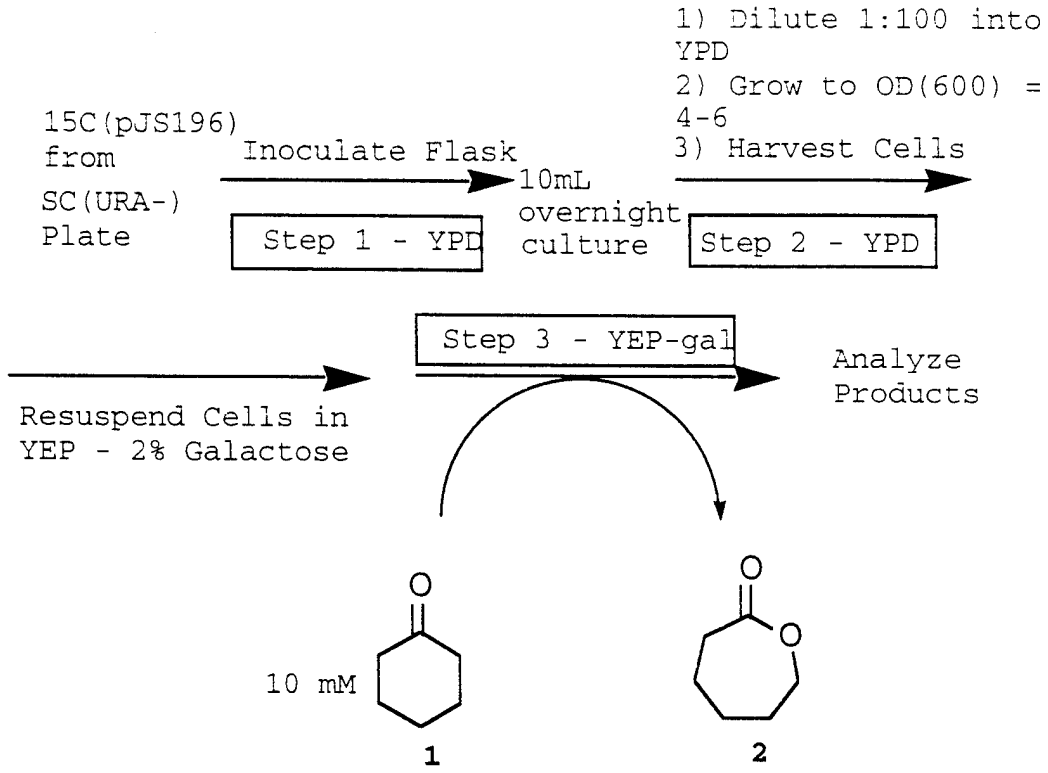
Scheme 2.7 Growth Strategy for Table 2.2

The following were varied during this sequence of reactions. First, we varied the initial cell mass used for the transformation from 1.6 to 10g. In addition, we varied the amount of galactose used for induction (0.2, 2.0, or 20%). All reactions were performed with the indicated concentration of galactose in 0.5% ammonium sulfate (table 2.2). The results from these studies showed that a galactose concentration of 2.0% gave the best results. However, the low extent of reaction and poor cell growth suggested that the use of richer growth medium for the biotransformation might be more suitable.

Table 2.2 Variation of Initial Cell Mass and Amount of Galactose

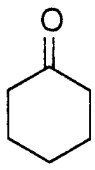
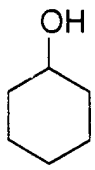
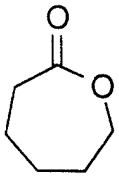
% galactose	Starting Cell Concentration (mg/mL)	Results
0.2%	16	No caprolactone formed
0.2%	32	*ca.* 15% caprolactone by NMR
2.0%	16	*ca.* 15% caprolactone by NMR
2.0%	100	*ca.* 20% caprolactone by NMR
20%	20	No caprolactone formed

Yeast-mediated Baeyer-Villiger oxidations in YEP-2% galactose. Based on our earlier results, we substituted YEP-gal for 0.5% ammonium sulfate in water for the biotransformation. Scheme 2.8 shows the sequence followed in these experiments. We were initially concerned that the complex growth media would complicate product isolation. However, a test extraction of the YEP-gal media with ethyl acetate showed that none of the water-soluble components in the YEP-gal media were extracted into the organic layer. Test reactions, both with and without 0.5% ammonium sulfate were performed and the amount of caprolactone formed was determined by GC analysis after 24 hours (table 2.3).



Scheme 2.8 Modified Sequence Using YEP-gal for Step 3

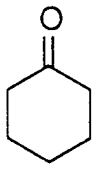
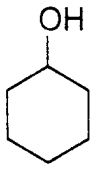
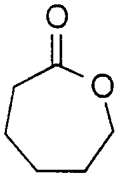
Table 2.3 Results from Experiments Outlined in Scheme 2.8

YEP-gal w/ or w/o $(NH_4)_2SO_4$	Cell conc. Wet weight (mg/mL)	% composition after 24 hours		
		 1	 3	 2
with	20	10	85	5
without	20	10	50	40

Based on the results in table 2.3, it was apparent that the ammonium sulfate caused the cells to grow too quickly,

which favored ketone reduction over the desired Baeyer-Villiger oxidation. We then conducted a series of experiments in which the initial amount of washed yeast cells was varied in order determine the optimum amount. All reactions were followed by GC and allowed to proceed for 48 or 96 hours (table 2.4).

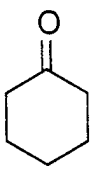
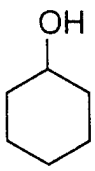
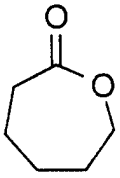
Table 2.4 Results from Scheme 2.8 with Variations in Starting Cell Mass

Initial Cell Conc. (mg/mL)	Reaction Time	% Composition		
		 1	 3	 2
0.5	96 hrs	0	12	88
1.5	96 hrs	0	10	89
5.0	96 hrs	0	14	85
10	96 hrs	6	21	73
20	48 hrs	8	46	46
400	48 hrs	42	43	15

After determining that starting concentrations of yeast cells of approximately 2mg/mL were optimal for bioconversions using 15C(pJS196), we altered the cell growth conditions in an effort to eliminate a lag in the Baeyer-Villiger oxidation that occurred in the early portion of the biotransformation. We hypothesized that this lag arose because the production of

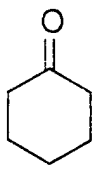
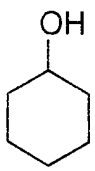
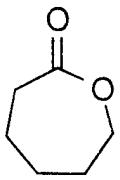
cyclohexanone monooxygenase was dependent on the change in metabolism that accompanied the change in carbon sources. For this reason, we diluted the overnight culture into YEP-gal, rather than YPD. After growing the cells for 24 hours in YEP-gal, they were harvested by centrifugation and used for the Baeyer-Villiger oxidation of cyclohexanone as before. As shown in table 2.5, this modification had little effect on the fraction of cyclohexanone converted to caprolactone.

Table 2.5 Attempt to Eliminate Lag in the Biotransformation

Plasmid	Initial Cell conc. (mg/mL)	Time (hours)	Media for Step 1	Media for Step 2	% Composition		
					 **1**	 **3**	 **2**
pJS196	1.5	72	YPD	YPD	0	22	68
pJS196	1.5	72	YPD	YEP-gal	0	26	64

Reasoning that poor expression of cyclohexanone monooxygenase was responsible for the poor conversions to the lactone product, we replaced 15C(pJS196) with 15C(pKR001). Using the same conditions, this biotransformation gave caprolactone as the major product, with only 4% of the ketone reduction product. The lactone product was isolated from this reaction in 73% yield (table 2.6).

Table 2.6 Biotransformations Using 15C(pKR001)

Plasmid	Initial Cell conc. (mg/mL)	Time (hours)	Media for Step 1	Media for Step 2	% Composition		
					 **1**	 **3**	 **2**
pKR001	2.0	46	YPD	YEP-gal	0	4	96
pKR001	2.2	20	YPD	None	0	2.5	97

While these alterations improved the yield of the lactone product and decreased the amount of reduction, the problem of plasmid retention remained. Plasmid retention for the 15C(pJS196) reactions ranged from 50-60% after two growth cycles in YPD. Under the same conditions with 15C(pKR001), the plasmid retention rate was about 30%. Finally, 15C(pKR001) grown for 1 cycle in YPD followed by a second cycle in YEP-gal gave a plasmid retention rate of less than 20%.

Since cultures grown under non-selective conditions gave rise to a sub-population that had lost the plasmid, we sought to minimize the number of sub-culturing steps between the initial inoculation and the biotransformation. We therefore developed a protocol that involved only one growth under non-selective conditions (scheme 2.9). This protocol has been

used for all of the preparative biotransformations reported in this thesis. These were performed in YEP containing 2% galactose, 10mM ketone and a starting cell concentration of 2mg/mL.

The recovered yield of caprolactone using the one-step growth reaction was 88%. In addition, the plasmid retention rate was 70%, compared to the earlier plasmid retention rate of less than 20% for the YPD, YEP-gal system. The growth of 15C(pKR001) was measured under these optimized conditions by the absorbence at 600nm as a measure of cell density (figure 2.5). Interestingly, the chemistry occurs well before the cells have reached their maximal density.

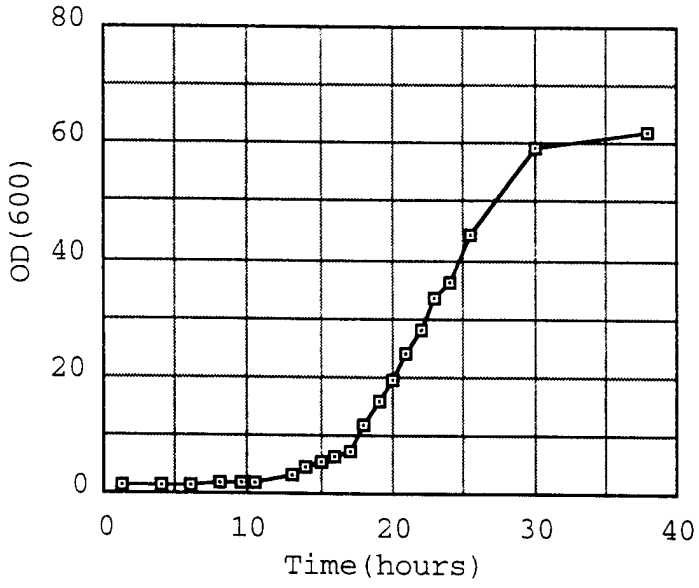
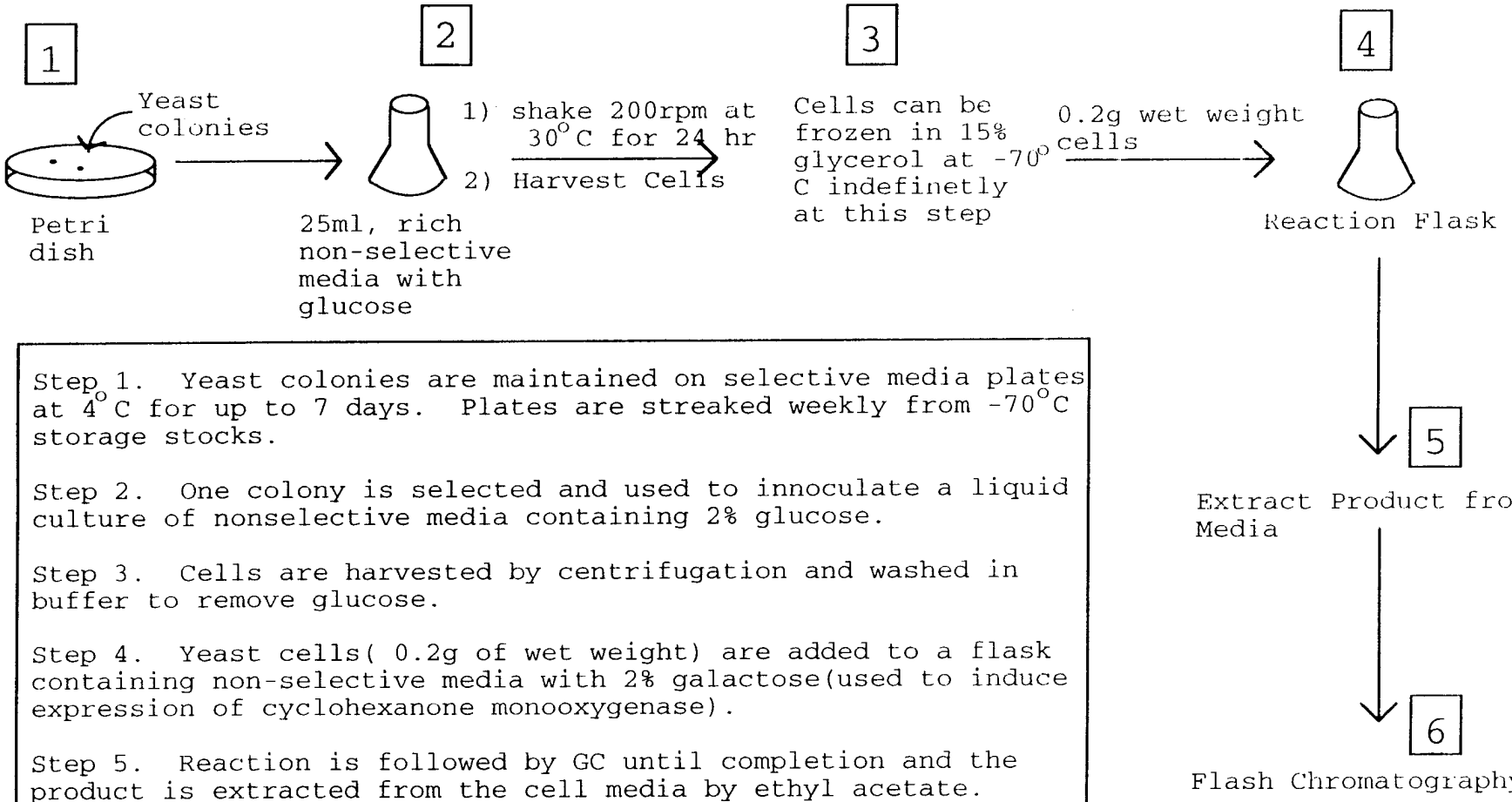


Figure 2.5 Growth Curve for 15C(pKR001)

Scheme 2.9 Final Reaction Procedure



Step 1. Yeast colonies are maintained on selective media plates at 4°C for up to 7 days. Plates are streaked weekly from -70°C storage stocks.

Step 2. One colony is selected and used to innoculate a liquid culture of nonselective media containing 2% glucose.

Step 3. Cells are harvested by centrifugation and washed in buffer to remove glucose.

Step 4. Yeast cells(0.2g of wet weight) are added to a flask containing non-selective media with 2% galactose(used to induce expression of cyclohexanone monooxygenase).

Step 5. Reaction is followed by GC until completion and the product is extracted from the cell media by ethyl acetate.

2.3 Control Experiments

General. After developing the best growth strategy for bioconversions using 15C(pKR001), we performed two control experiments. One control was conducted to ensure that the Baeyer-Villiger oxidation was not catalyzed by a yeast enzyme, rather than by cyclohexanone monooxygenase. A second control experiment verified that the expression of the cyclohexanone monooxygenase gene could be controlled by the inclusion of galactose in the media.

Host cells that lack expression plasmid. Yeast 15C, with no expression plasmid, was grown in the presence of 10mM cyclohexanone for 120 hours under our standard conditions. The reaction was followed by GC. After 120 hours, no caprolactone was present, but most of the cyclohexanone had been reduced to cyclohexanol (figure 2.6).

Attempted biotransformation in the absence of gene expression. To determine whether the galactose promoter controlling cyclohexanone monooxygenase in pKR001 responded to the carbon source, two reactions were conducted: one in which the yeast cells 15C(pKR001) were induced by the addition of 2% galactose and the other in which the cells were grown in the presence of 2% glucose (figure 2.7). The results showed no caprolactone production in 24 hours in the YPD medium, while the reaction went to completion in 23 hours

in the YEP-galactose media. Clearly, the promoter responded to control by the presence or absence of galactose.

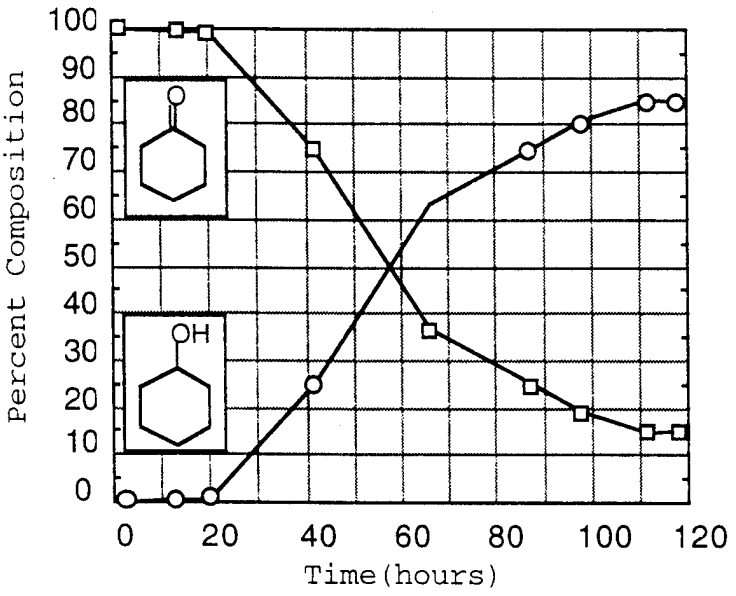


Figure 2.6 Host Cells That Lack Expression Plasmid

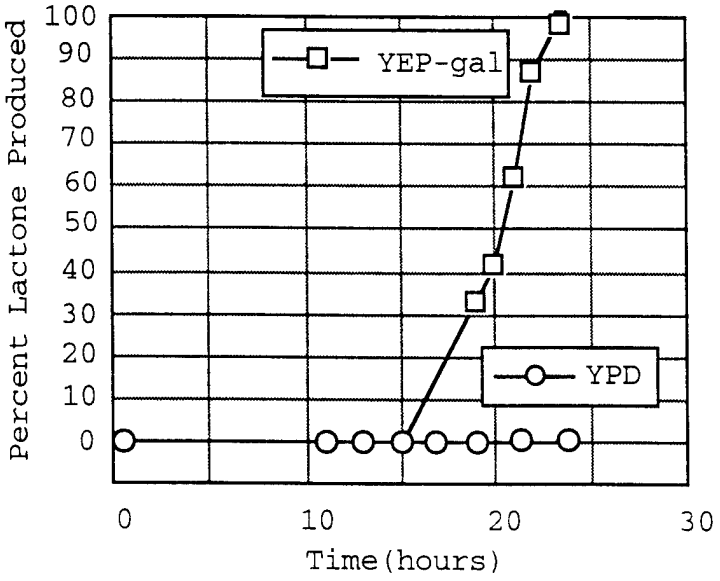
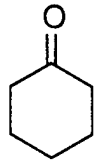
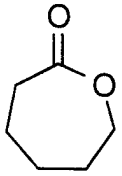
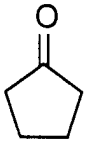
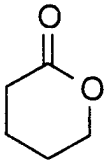


Figure 2.7 Attempted Biotransformation in the Absence of Gene Expression

2.4 Oxidations of Unsubstituted Cyclic Ketones

General. Cyclohexanone and cyclopentanone were chosen for our initial studies for several reasons. Both substrates are symmetrical and oxygenation does not induce chirality. In addition, cyclohexanone has been studied extensively with this enzyme. Table 2.6 contains the isolated yields of these substrates under our standard conditions. Figures 2.8 and 2.9 show the reaction progress as a function of time.

Table 2.6 Yields for Unsubstituted Cyclic Ketones

Substrate	Product	Yield	Completion Time (hours)
 **1**	 **2**	88%	20
 **4**	 **5**	83%	48

Cyclopentanone. Our studies showed that cyclopentanone was consumed more slowly than cyclohexanone under our reaction conditions. Fortunately, cyclopentanone was not as susceptible to the host cell reductases as cyclohexanone allowing us to start with a slightly higher cell concentration (5mg/mL) and thereby reduce the reaction time. A short reaction time was critical to obtain high yields of δ-valerolactone.

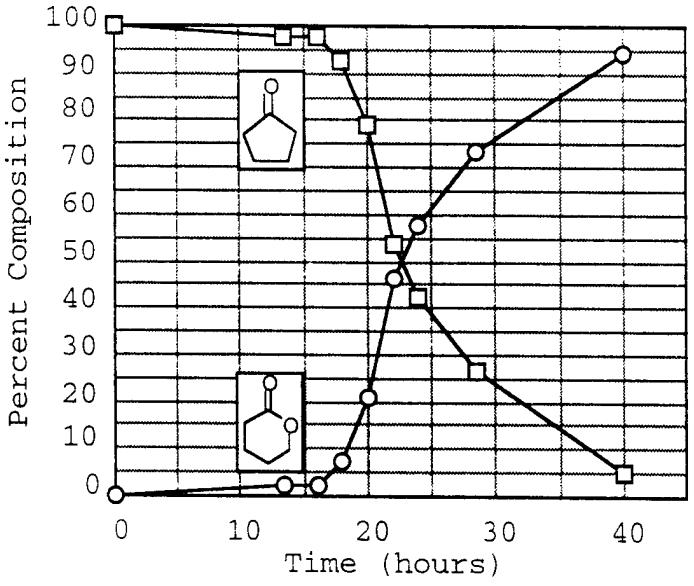


Figure 2.8 Time Course for the Baeyer-Villiger Oxidation of Cyclopentanone

Cyclohexanone. As discussed previously, our optimized conditions afforded an 88% yield of caprolactone with very little ketone reduction.

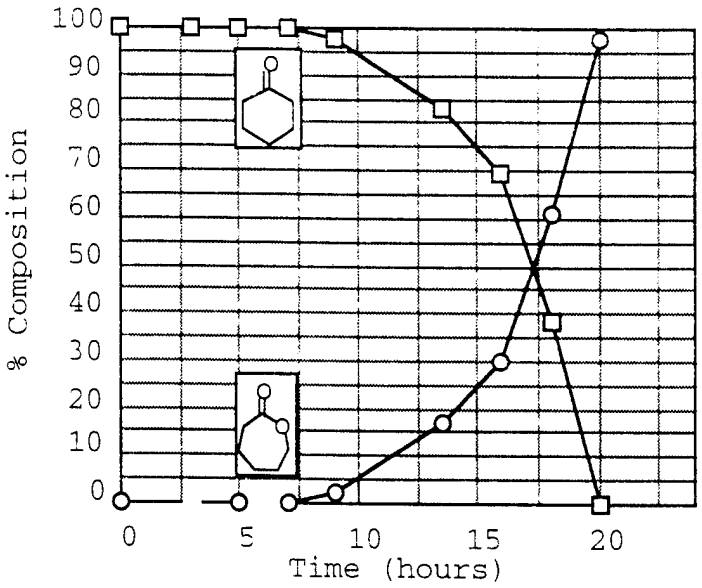


Figure 2.9 Time Course for the Baeyer-Villiger Oxidation of Cyclohexanone

Constitutive expression of cyclohexanone monooxygenase. We briefly explored the constitutive expression system present in 15C(pKR002). The transformed yeast, 15C(pKR002), gave satisfactory results under standard reaction conditions. Completion time for the standard 10mM cyclohexanone reaction was 23 hrs. However, plasmid retention rates were very low: (less than 10% compared to greater than 70% for 15C(pKR001) after overnight growth in non-selective media (YPD). We surmised that the metabolic drag created by the high-level, constitutive gene expression slowed the growth of cells containing pKR002. This slower growth rate allowed the sub-population of yeast cells that had spontaneously lost the plasmid to over-grow and dominate the culture grown under non-selective conditions. To test this theory, four biotransformations were performed. In the first, the yeast

were grown overnight in non-selective media (YPD) and the biotransformation was performed in selective media (SC, Trp-). In the second, the yeast were grown in selective media (SC,Trp-) and biotransformation was also performed in selective media. In the third strategy, the yeast were initially grown in non-selective media and the biotransformation was performed in selective media. Finally, the standard conditions were used, in which both the pre-culture and the biotransformation were both performed in non-selective media. The results of these four strategies are shown in figure 2.10.

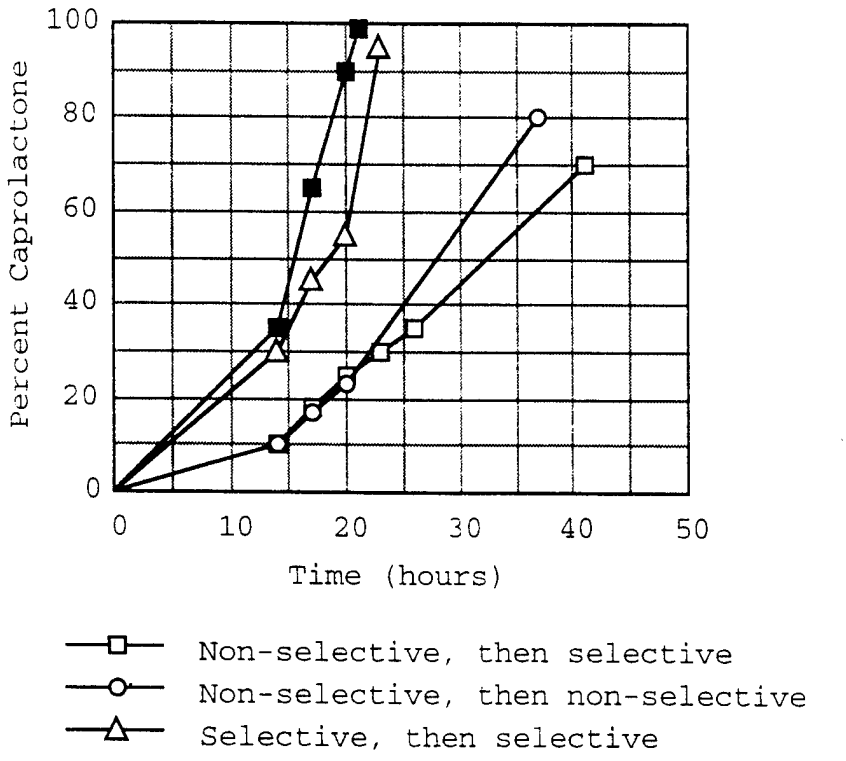
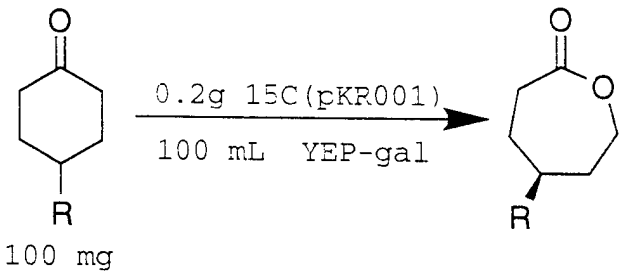


Figure 2.10 Constitutive Expression System Results

The results clearly showed that the best way to perform the biotransformation of cyclohexanone using 15C(pKR002) was to grow the cells in selective media from the plate, followed by performing the reaction in non-selective media (YPD). Although these results appear to be comparable to the 15C(pKR001) biotransformation, there are some significant differences. In particular, the plasmid retention was less than 20% at the end of the reaction. This should be compared to the plasmid retention of greater than 70% for 15C(pKR001). For this reason, cultures of 15C(pKR002) ceased to catalyze the Baeyer-Villiger oxidation at detectable rates after 48 hours.

2.5 Oxidations of 4-substituted Cyclohexanones

A series of 4-substituted cyclohexanones was chosen to test the stereospecificity of cyclohexanone monooxygenase in our yeast expression system. All reactions were carried out following the procedure outlined in scheme 2.10 and the results are shown in table 2.7. All of the substrates were prochiral with the product lactone being optically active. Previous studies have shown that this enzyme gives values of enantiomeric excess between 90 and 98% with these substrates (Taschner et al., 1988, 1991, 1992a, 1993).



Scheme 2.10 General Biotransformation Procedure

4-methylcyclohexanone **6** was easily oxidized under our standard conditions to afford the (*S*)-lactone in high optical purity. On the other hand, the Baeyer-Villiger oxidation of 4-ethylcyclohexanone **8** was markedly slower, requiring 100 hours to reach 90% completion. Reasoning that the more lipophilic substrate might be toxic to the yeast cells, we explored the use of stoichiometric quantities of β-cyclodextrin (relative to the ketone)(Bar et al, 1989; Goetschel and Bar, 1991; Hesselink et al., 1989; Jadoun et al., 1993a, 1993b; Singer et al., 1991). In the case of 4-methylcyclohexanone, the addition of β-cyclodextrin did not markedly affect the rate of biotransformation (figure 2.11). On the other hand, β-cyclodextrin dramatically improved the yeast mediated Baeyer-Villiger oxidation of ketone **8** (figure 2.12).

Table 2.7 Results of 4-substituted Cyclohexanone Reactions

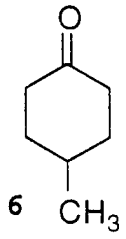
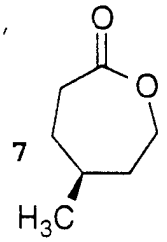
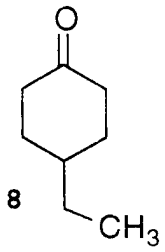
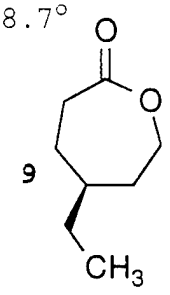
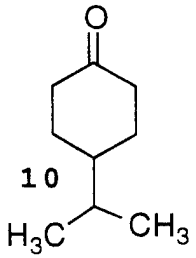
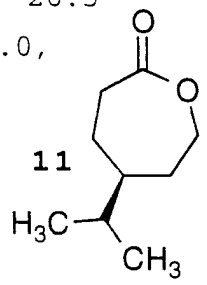
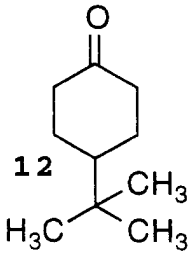
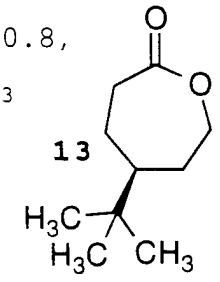
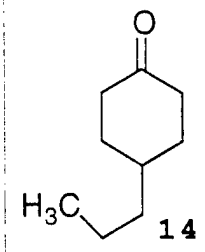
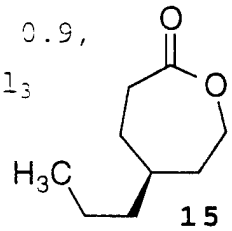
Substrate	Product	Yield	Reaction Time (hr)	% ee
 **6**	$[\alpha]_D = -44.9°$ c = 1.4, $CHCl_3$  **7**	83	21	>98
 **8**	$[\alpha]_D = -28.7°$ c = 3.2, $CHCl_3$  **9**	74	21	>98
 **10**	$[\alpha]_D = -26.5°$ c = 2.0, $CHCl_3$  **11**	50	50	>98
 **12**	$[\alpha]_D = -41.6°$ c = 0.8, $CHCl_3$  **13**	47	90	>98

Table 2.7 Continued

Substrate	Product	Yield	Reaction Time (hr)	% ee
 14	$[\alpha]_D = -22.1°$ c = 0.9, $CHCl_3$  15	63	70	92

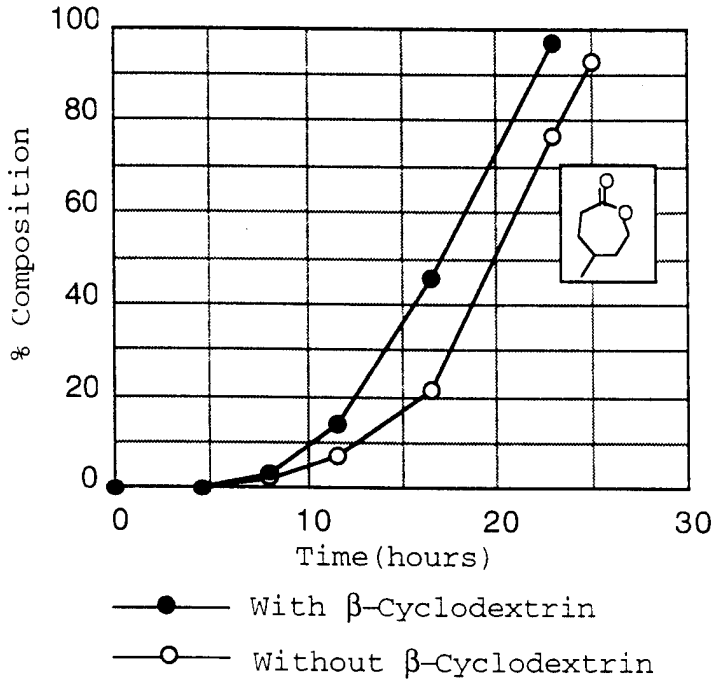


Figure 2.11 Time Course for the Yeast-mediated Oxidation of 4-methylcyclohexanone.

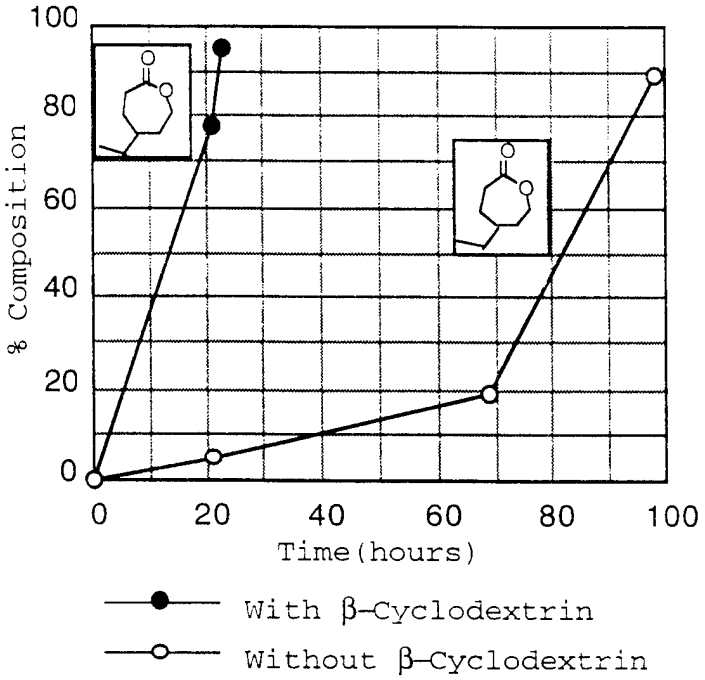


Figure 2.12 Time Course for the Yeast-mediated Oxidation of 4-ethylcyclohexanone

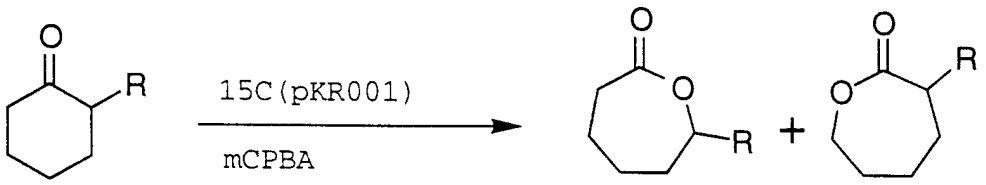
Substrates 4-*iso*-propylcyclohexanone **10**, 4-*tert*-butylcyclohexanone **12**, and 4-*n*-propylcyclohexanone **14** required the addition of one equivalent of β-cyclodextrin for the reaction to proceed. Without β-cyclodextrin, these substrates were toxic to the cells: samples of the reaction mixture taken after 24 hours and plated on YPD plates showed no cell viability. The addition of β-cyclodextrin allowed the cells to grow normally. In addition, the β-cyclodextin appeared to help increase the solubility of these lipophilic substrates. The optical purities of the products were determined by chiral-phase GC analysis. As expected, our results were virtually identical to those obtained from

reactions using purified enzyme (Taschner and Black, 1988; Taschner et al., 1993).

2.6 Oxidations of 2-substituted Cyclohexanones

General. A series of 2-substituted cyclohexanones were chosen as our next substrates. Surprisingly, these have not been assessed as substrates for *CHMO*. This series of compounds introduced issues of both regio- and enantioselectivity that have not previously been addressed.

Regioisomer determination. To answer the question of regioselectivity, each of these substrates was transformed to its lactone product using both *m*-CPBA and 15C(pKR001). The products were analyzed by chiral capillary GC, IR, ^{1}H NMR and ^{13}C NMR.

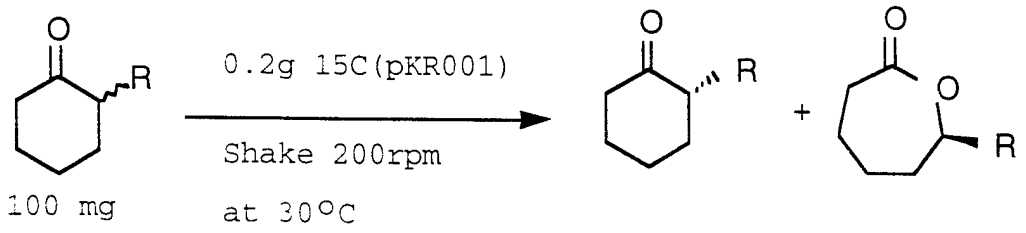


Scheme 2.11

We performed this procedure with the 2-methyl-, 2-ethyl-, 2-*n*-propyl- and 2-*n*-butylcyclohexanones. In the synthetic reactions we saw no evidence of another regioisomer by capillary GC analysis or by ^{13}C NMR. In the biotransformations, only the 2-ethylcyclohexanone showed another peak that we have tentatively assigned as the "wrong"

regioisomer. The non-standard regioisomer comprised less than 5% of the product mixture at 50% completion.

Enantioselectivity determination. After analysis for regioselectivity, we then evaluated each of the substrates for enatioselectivity and the ability to kinetically resolve the enantiomers following scheme 2.12.



Scheme 2.12 2-substituted Cyclohexanone Biotransformations

The kinetic resolution (E) of racemic substrates can be described in terms of the relative rates at which each enantiomer is converted to product. Equation 2.1 describes this relationship as the ratio of the two second order rate constants for the (*R*)- and (*S*)- ketone enantiomers (Chen, et al., 1982).

Equation 2.1

$$\frac{\ln(S/S_0)}{\ln(R/R_0)} = \frac{V_S/K_S}{V_R/K_R} = E$$

Here, E is the ratio of the specificity constants and V_S, K_S, V_R, and K_R denote the maximal velocities and the Michaelis constants of the (*S*)- and (*R*)- enantiomers, respectively. Using these equations, one can develop a plot that graphically relates the enantiomeric purity of the remaining substrate to the extent of conversion (figure 2.14)(Chen, et al., 1982).

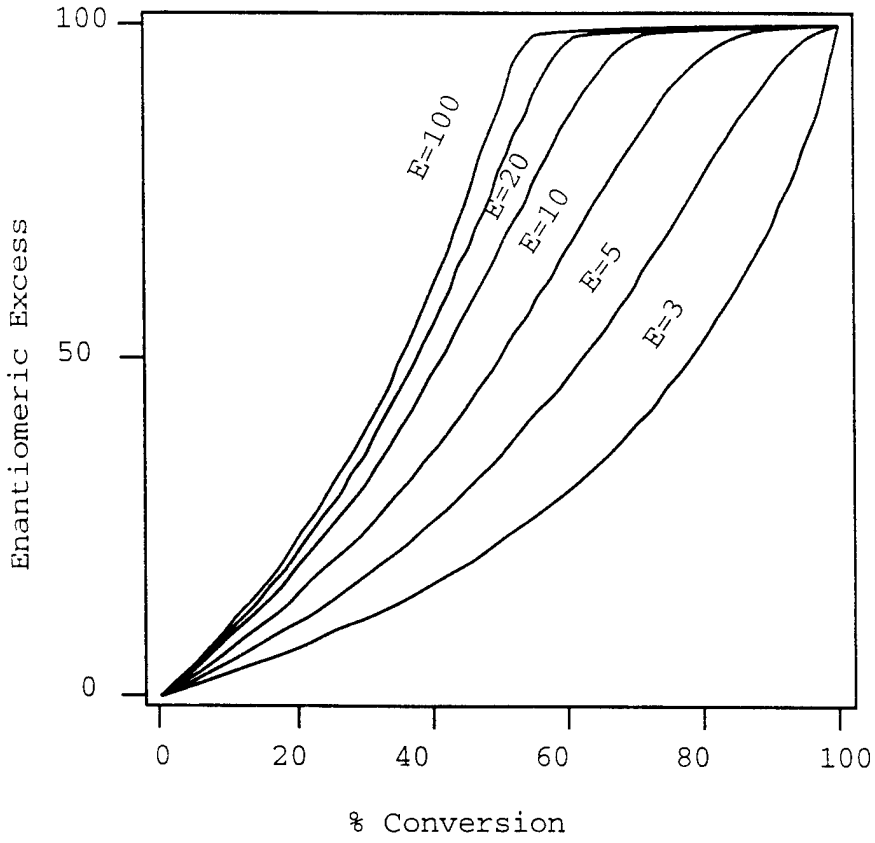


Figure 2.14 Theoretical E Plots

By visual comparison of experimental data to a family of theoretical curves, the E of a reaction can be estimated. A

more quantitative estimate of this value can be determined by a non-linear fit of the experimental data to equation 2.2.

Equation 2.2

$$y = \left[\frac{(1+x)^{E}}{(1-x)} \right]^{\frac{1}{(1-E)}}$$

x = ee of the remaining ketone

y = fractional conversion

The biotransformations were followed by GC in order to detect noticeable rate differences as one enantiomer is consumed. A biphasic curve would be indicative of differing rates for the two ketone enantiomers. Racemic 2-methylcyclohexanone **16** showed only a small rate difference between the two starting enantiomers shown in time course (figure 2.15). However, by re-analyzing the data, and performing a non-linear fit to equation 2.1, an E value of 10 was obtained (figure 2.16)

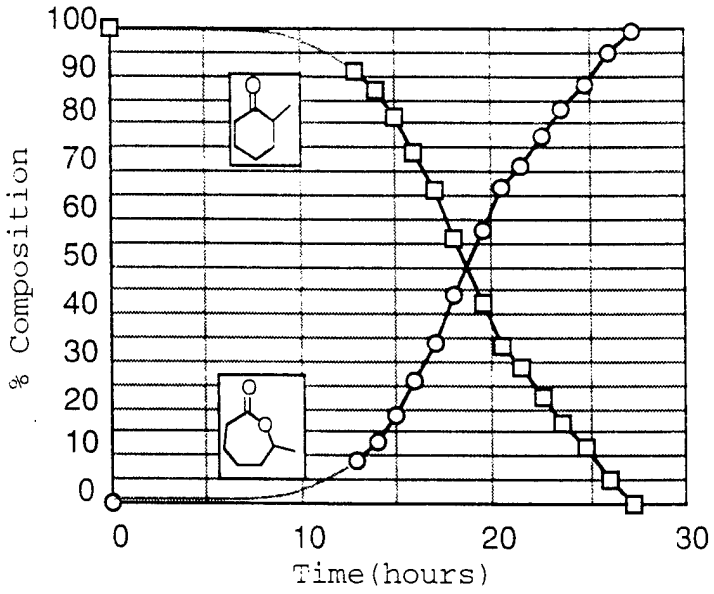


Figure 2.15 Time Course for the Yeast-mediated Oxidation of 2-methylcyclohexanone

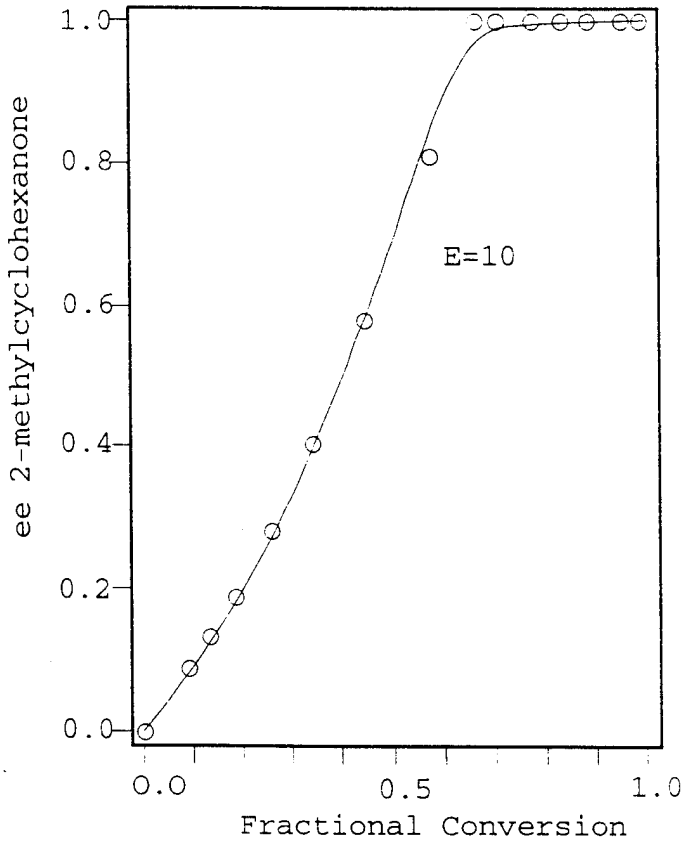


Figure 2.16 Determination of E for the Yeast-mediated Baeyer-Villiger Oxidation of 2-methylcyclohexanone

This illustrates that even though a rate change may be minimal or non-existent when following a simple time course, there may infact be a very large rate difference. This E plot allows one to calculate when best to stop the reaction to achieve the best ee value for the ketone or the lactone. In the case of ketone **16**, stopping the reaction at approximately 60% completion would yield optically pure enriched ketone.

Racemic 2-ethylcyclohexanone **18** showed a distinct biphasic curve in the time course, indicating two different rates for the enantiomers (figure 2.17). Once again, while dramatic, the rate change on the time course plot was not indicative of the true magnitude of E.

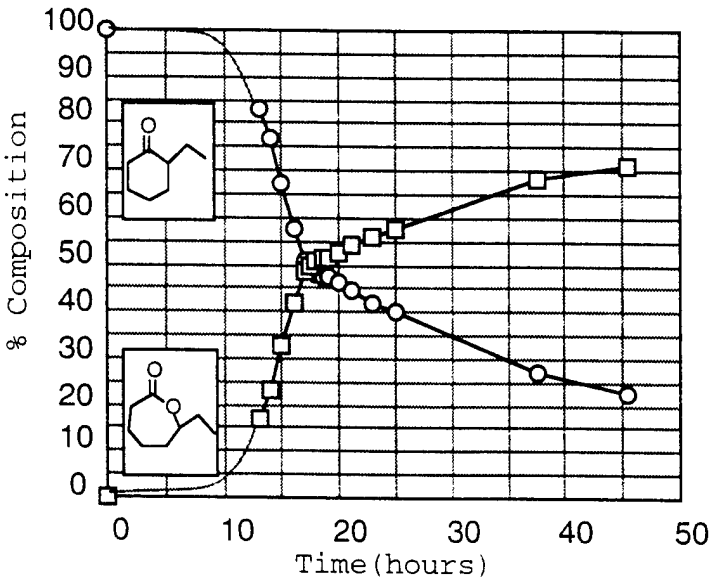


Figure 2.17 Time Course for the Yeast-mediated Oxidation for 2-ethylcyclohexanone

With substrate **18**. the E value was equal to 200 (figure 2.18). If one stops the reaction at 50%, the resulting enantiomericlly enriched ketone and lactone products have ee values between 96 and 98%. By stopping the reaction slightly before or slightly after 50% completion, the ee of ketone or the lactone can be maximized.

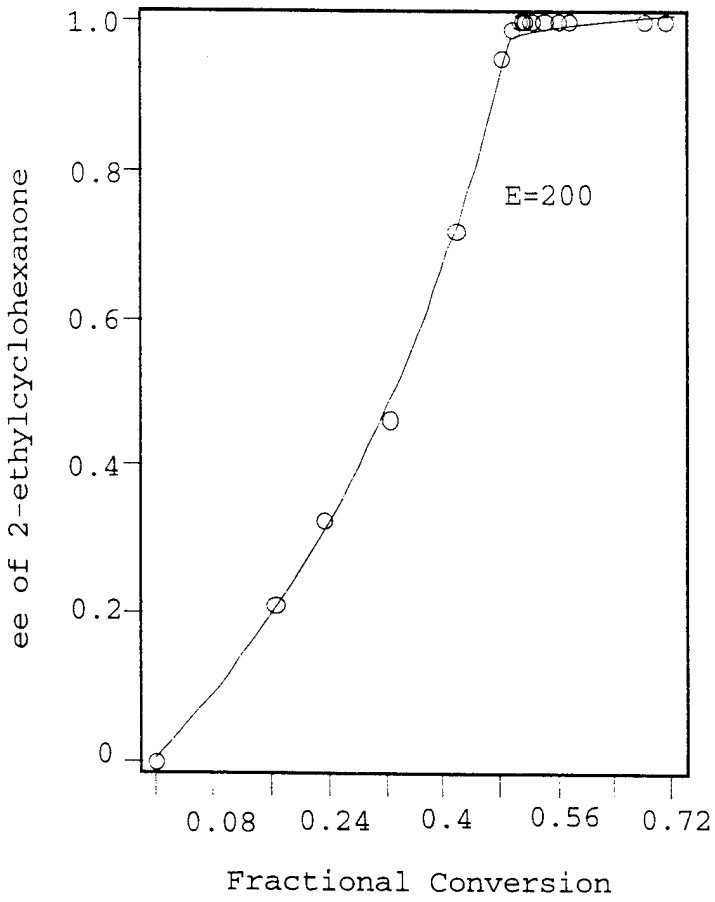


Figure 2.18 Determination of the E for the Yeast-mediated Baeyer-Villiger Oxidation of 2-ethylcyclohexanone

Racemic 2-*n*-propylcyclohexanone **20** and 2-*n*-butylcyclohexanone **22** were also oxidized using 15C(pKR001). Time course plots of the substrates showed conversion to 50%

completion followed by a second rate attributable only to the reduction of the remaining ketone by host yeast reductases. This result indicated that *CHMO* was consuming only one of the starting ketone enantiomers. E-plots of the bioconversions of substrates **20** and **22** confirmed an E value of greater than 200 for each of these substrates (figure 2.19).

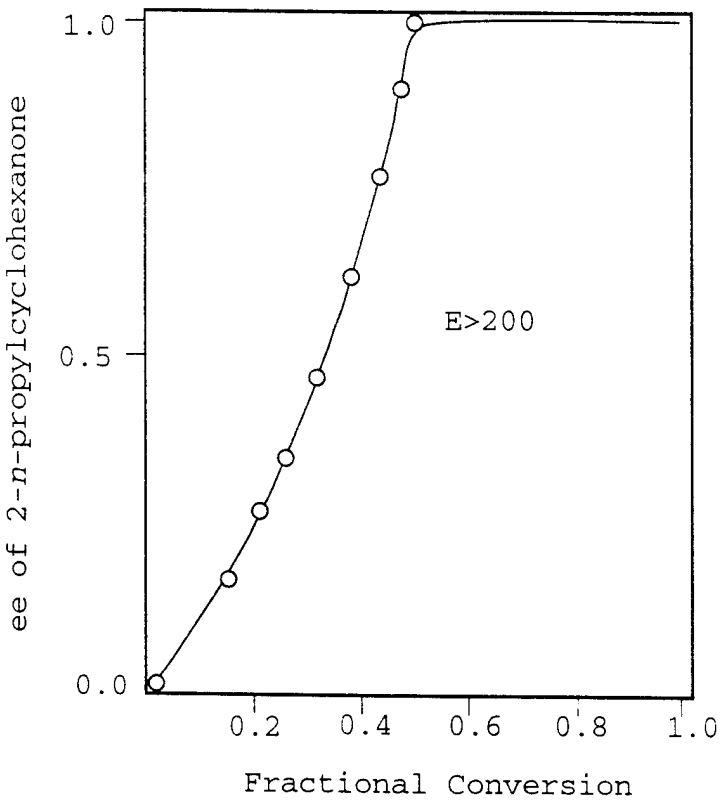


Figure 2.19 Determination of E for the Yeast-mediated Baeyer-Villiger Oxidation of 2-*n*-propylcyclohexanone

Preparative scale reactions were then done on substrates **16**, **18**, **20** and **22** (scheme 2.12). The oxidations were stopped at 50% conversion and the resulting lactones and ketones were purified. The absolute configuration of (*R*)-3-methylcaprolactone and 2-ethylcyclohexanone **18** are known

(Fouque and Rousseau, 1989; Saigo, et al., 1983). Our results (table 2.9), confirmed that the (*S*) isomer was the faster reacting substrate. With substrates **20** and **22**, only the (*S*) isomer was produced.

Table 2.9 Results of 2-substituted Cyclohexanone Reactions

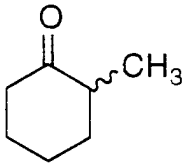
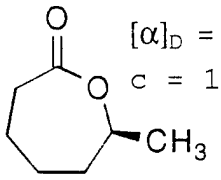
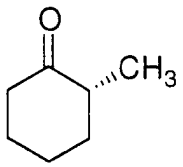
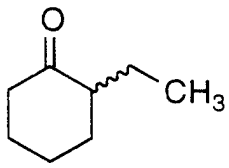
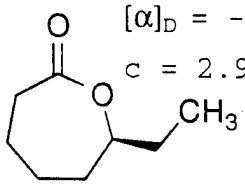
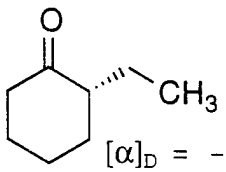
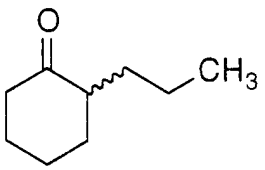
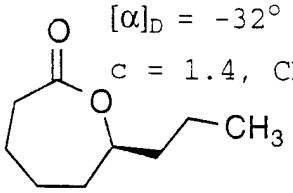
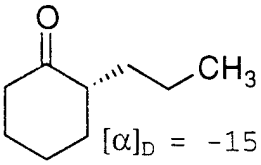
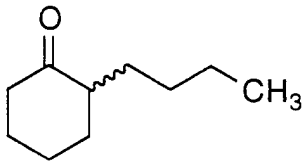
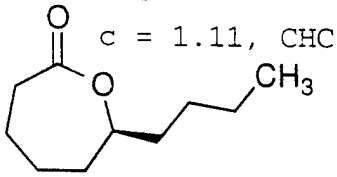
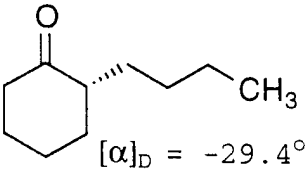
Substrate	Product	E	Yield	ee
 **16**	 $[\alpha]_D = -6.5°$ c = 1.8, $CHCl_3$ **(*S*)-17**	10	ND	49%
	 **(*R*)-16**		ND	ND
 **18**	 $[\alpha]_D = -37.1°$ c = 2.9, $CHCl_3$ **(*S*)-19**	200	79%	>98%
	 $[\alpha]_D = -23.8°$ c = 3.0, $CHCl_3$ **(*R*)-18**		69%	94%

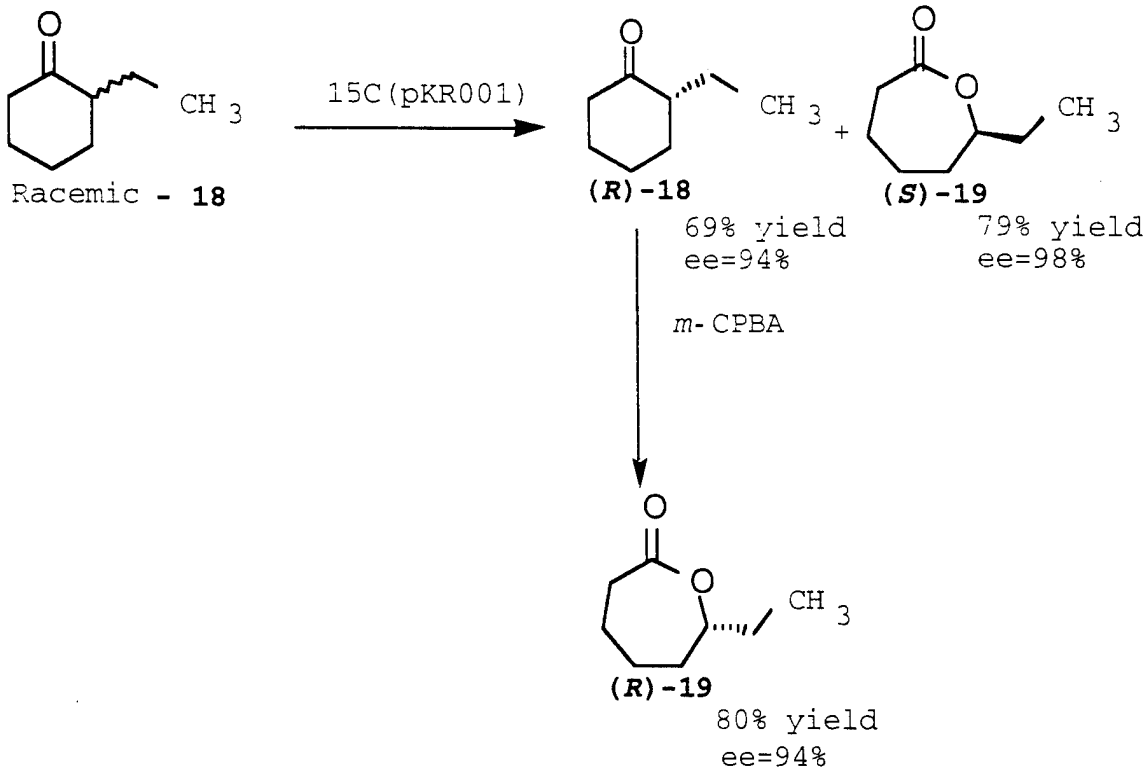
Table 2.9 Continued

Substrate	Product	E	Yield	ee
 **20**	 $[\alpha]_D = -32°$ c = 1.4, $CHCl_3$ **(*S*)-21**	200	54%	>98%
	 $[\alpha]_D = -15.3°$ c = 1.6, $CHCl_3$ **(*R*)-20**		66%	>90%
 **25**	 $[\alpha]_D = -18.6°$ c = 1.11, $CHCl_3$ **(*S*)-23**	200	59%	>98%
	 $[\alpha]_D = -29.4°$ c = 1.17, $CHCl_3$ **(*R*)-22**		64%	90%

In order to show the versatility of this system, we prepared both (*S*)- and (*R*)- 3-ethylcaprolactone **(*S*)-19**, **(*R*)-19** from a single biotransformation (scheme 2.13). Substrate **18** was oxidized with 15C(pKR001) to 50% completion and both the remaining (*R*)-ketone and the (*S*)-lactone product were

isolated. The (*R*)-ketone was then oxidized using *m*-CPBA to give the (*R*)-lactone in 56% overall yield. Similar procedures could also be used to isolate both lactone isomers from the remaining substrates in table 2.9.

Scheme 2.13 2-ethylcyclohexanone Reaction



2.7 Conclusions

We have been able to successfully clone and express cyclohexanone monooxygenase in yeast. The expressed enzyme appears to have the same substrate specificity and enantioselectivity as the purified enzyme and whole cell *Acinitobacter* sp. systems. Yeast reduction of the starting

ketones was minimal under our conditions. In addition, there are very few cell metabolites that are extracted into the organic solvent, giving very clean reaction products. This system is easy to use and can be used by an organic chemist as an off-the-shelf chiral peracid for Baeyer-Villiger reactions.

Since we have created a library of expression plasmids, each expressing the *CHMO* gene at a different levels, there now exists a framework in which other chemically useful enzymes can be expressed. Toxic enzymes can be inserted into the pM949 expression plasmid, while relatively innocuous enzymes can be expressed in pYES2 and pG-3 in progressively higher levels.

We have been able to show that pro-chiral 4-substituted cyclohexanones are transformed by the engineered yeast only to the (*S*)-lactone. In addition, any problems caused by highly lipophilic compounds can be easily solved by the addition of cyclodextrins.

Lastly, we have shown that racemic 2-substituted cyclohexanones can be kinetically resolved using the engineered yeast. We have shown that, one can start with racemic 2-substituted cyclohexanones, and in two steps, produce both enantiomers of the 3-substituted caprolactones in relatively high yields.

CHAPTER 3
EXPERIMENTAL

3.1 General - Organic Synthesis

General. Proton NMR spectra were taken on a General Electric QE-300, Varian Gemini or VXR-300 instrument operating at 300 MHz. All spectra were obtained in $CDCl_3$ and referenced to residual $CHCl_3$(7.26 ppm). ^{13}C NMR spectra were taken on a Varian Gemini 300 operating at 300 MHz in $CDCl_3$ and were referenced to solvent (76.0). IR spectra were recorded from thin films on a Perkin-Elmer 1600 FT-IR spectrophotometer. Optical rotations were measured from chloroform solutions using a Perkin-Elmer 241 polarimeter operating at ambient temperature. Packed column gas chromatography was performed on a Hewlett-Packard 5710 equipped with a flame ionization detector and a 0.3 X 300 cm column of 10% OV-17 on Chromosorb WHP with helium as carrier gas. Two oven temperature gradients were used: A, 80°C (4 min) to 220°C(4 min) at 16°C/min; B 110°C(2 min) to 230°C (8 min) at 8°C/min. Capillary gas chromatography was performed on a Hewlett-Packard 5880A or 5890A equipped with a flame ionization detector and a 0.32mm x 30m DB-17 column for non-chiral separations and a Chrompack 0.25mm x 25m CP chirasil-Dex CB column for chiral separations. Gradient used for the

DB-17 column: 80°C (5 min) to 180°C (5 min) at 10°C/min. Gradient used for the chiral column: 110°C (2 min) to 140°C (5 min) at 1°C/min followed by 10°C/min to 180°C (5 min). Thin layer chromatography was performed on Silica Gel 60. Reaction products were purified by flash chromatography (Still et al., 1978) using 60Å silica gel and 1:1 ether:hexanes as the mobile phase.

All reagents were obtained from commercial suppliers and were used as received. Cyclodextrins were a generous gift of Amaizo (Hammond, IN).

Production of racemic lactone standards. Lactones used as authentic standards were prepared in the following manner. Lactones **2**, **5**, **7**, **9**, **11**, **13**, **15**, **17**, **19**, **21**, and **23** were prepared using a modified protocol from (Koch and Chamberlin, 1989). The appropriate ketone (0.1g) was dissolved in 10mL of $CHCl_3$ at room temperature. *m*-CPBA (1.5 eqv) was then added to the stirred solution. The reaction was followed by GC and allowed to reach completion. Reaction times varied between 12 hours and 48 hours. After the reaction was complete, the organic layer was washed 3 times with an equal volume of saturated sodium bicarbonate solution. The organic layer was then removed by rotary evaporation and the crude material was purified by flash chromatography. Structures were verified by GC-MS, IR, ^{1}H NMR, and ^{13}C NMR when appropriate.

Preparation of 2-ethylcyclohexanone.

2-Ethylcyclohexanone was not available commercially and was synthesized from 2-ethylcyclohexanol using pyridinium chlorochromate (Corey and Suggs, 1975). In a 500mL round bottom flask, 0.15 moles(3.23 g) of pyridinium chlorochromate (PCC) was suspended in 50mL of CH_2Cl_2. A 0.1 mole(1.28g) portion of 2-ethylcyclohexanol, dissolved in 10mL of CH_2Cl_2, was added to the stirred flask in one portion. The reaction was stirred at room temperature for 2 hours, when TLC analysis indicated that the oxidation was complete. The reaction mixture was diluted with 50mL of ethyl ether and the suspension was filtered. The remaining black solid was washed 3 times with 10mL of ethyl ether. The organic portions were combined, the solvent was distilled off and the residual green liquid was distilled to yield the pure ketone (0.77g, 61% yield).

2-ethyl cyclohexanone: ^1H NMR ($CDCl_3$, 300 MHz): δ = 2.2-2.4 (1H, m), 2.0-2.2 (2H, m), 1.6-1.9 (6H, m), 1.2-1.4 (2H, m), 0.9 (3H, t, 7.5 Hz) ; IR (film): 1710, 1450, 1207, 1124, 1055, 735, 709, cm^{-1}.

3.2 Spectral Data for Lactones

ε-Caprolactone: ^1H NMR ($CDCl_3$, 300 MHz): δ 4.2 (2 H, m), 2.6 (2H, m), 1.7-1.9 (6H, m); IR (film): 1729, 1341, 1239, 1148, 1078, 1055, 982, 930, cm^{-1}.

δ-Valerolactone: ^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.3 (2H, m), 2.5 (2H, m), 1.8-1.9 (4H, m); IR (film): 1729, 1341, 1239, 1158, 1078, 1055, 982, 930, cm^{-1}.

3-methylcaprolactone: ^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.4-4.5 (1H, m), 2.6-2.7 (2H, m), 1.9-2.0 (3H, m), 1.5-1.7 (3H, m), 1.3-1.4 (3H, d, 6.6 Hz); IR (film): 1726, 1450, 1281, 1240, 1178, 1074, 1016, 985, 938, 859, 694, cm^{-1}.

3-ethylcaprolactone: ^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.05-4.1 (1H, m), 2.6-2.7 (2H, m), 1.9-2.0 (3H, m), 1.5-1.7 (5H, m), 1.0 (3H, t, 7.2 Hz); IR (film): 1720, 1455, 1283, 1255, 1179, 1104, 1012, 912, 732,647, cm^{-1}.

3-*n*-propylcaprolactone: ^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.2-4.3 (1H, m), 2.6-2.7 (2H, m), 1.9-2.0 (3H, m), 1.4-1.7 (7H, m), 0.9 (3H, t, 7.2 Hz); IR (film): 1721, 1439, 1285, 1257, 1178, 1015, 912, 732, cm^{-1}.

3-*n*-butylcaprolactone: ^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.2-4.2 (1H, m), 2.5-2.8 (2H, m), 1.9-2.0 (3H, m), 1.2-1.8 (9H, m), 0.8-0.9 (3H, t, 6.9 Hz); IR (film): 1725, 1448, 1329, 1281, 1251, 1175, 1084, 910, 733, 647, cm^{-1}.

5-methylcaprolactone: ^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.2-4.4 (2H, m), 2.6-2.8 (2H, m), 1.5-2.0 (5H, m), 1.1 (3H, d, 6.6 Hz); IR (film): 1734, 1450, 1390, 1338, 1282, 1164, 1079, 1008, 980, 935, 862, 733, cm^{-1}.

5-ethylcaprolactone: ^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.1-4.4 (2H, m), 2.5-2.7 (2H, m), 1.8-2.0 (2H, m), 1.4 (1H, m), 1.2-1.4 (4H, m), 0.7-0.9 (3H, t, 7.2 Hz); IR (film): 1731, 1461, 1396, 1337, 1290, 1232, 1179, 1079, 914, 731, cm^{-1}.

5-*iso*-propylcaprolactone: ^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.0-4.4 (2H, m), 2.5-2.7 (2H, m), 1.8-2.0 (3H, m), 1.3-1.6 (4H, m), 1.8 (5H, m); IR (film): 1725, 1472, 1437, 1390, 1337, 1296, 1102, 862, cm^{-1}.

5-*n*-propylcaprolactone: ^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.1-4.4 (2H, m), 2.6-2.7 (2H, m), 1.9-2.0 (2H, m), 1.4-1.6 (4H, m), 1.2-1.4 (3H, m), 0.9 (3H, t, 7.2 Hz); IR (film): 1725, 1390, 1290, 1172, 1102, 1072, cm^{-1}.

5-*tert*-butylcaprolactone: ^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.1-4.4 (2H, m), 2.5-2.7 (2H, m), 1.8-2.0 (2H, m), 1.4 (1H, m), 1.2-1.4 (4H, m), 0.7-0.9 (3H, t, 7.2 Hz); IR (film): 1730, 1476, 1397, 1338, 1266, 1195, 1079, 862, 731,701, cm^{-1}.

3.3 General - Biochemistry and Molecular Biology

Chemicals and media. Rich medium (LB) was used for routine growth of bacteria. Recipe per liter, 10g Bactotryptone, 5g Yeast Extract, 10g NaCl. When appropriate, ampicillin was added to 200 μg/ml and chloramphenicol was used at concentrations of 170 μg/ml. For plates, 15g of agar per liter was added for plates.

Non-selective yeast medium (YPD) was used for routine maintenance of yeast strains. Recipe per liter, 10g Yeast Extract, 20g Bactopeptone, 20g Dextrose. For plates, 20g per liter of agar was added (Guthrie and Fink, 1991).

Selective yeast medium (SC) was used for routine maintenance of recombinant yeast strains. Recipe per liter,

6.7g Bacto-Yeast Nitrogen Base without amino acids, 20g Dextrose, 20mg L-Tryptophan, 20mg L-Histidine, 30mg L-leucine, 2mg Uracil. The appropriate amino acid or uracil was deleted for selective growth. For plates, 20g of agar per liter was added.

For short term storage, yeast and bacterial colonies were kept on plates sealed with parafilm at 4^0C. Yeast cells stored on plates lose activity rapidly after 7 days. For long term storage, strains were stored at -70^0C in 15% glycerol in TE buffer.

DNA manipulations. Restriction nucleases were purchased from New England Biolabs (Beverly, MA) and Promega (Madison, WI). T4 DNA polymerase was obtained from New England Biolabs. Yeast expression vector pYES2 was obtained from Invitrogen (San Diego, CA). Yeast expression vector pG-3 was a generous gift of Keith Yamamoto at UCSB (Schena et al., 1991). *E. coli* expression vector pET-21(+) was purchased from Novagen (Madison, WI). Recombinant DNA procedures were carried out essentially as described by Sambrook, et al., (1989). Oligonucleotides were obtained from Integrated DNA Technologies, Inc. (Coralville, IA).

DNA sequencing was accomplished using the Silver Sequence Kit (Promega) according to the manufacturer's instructions. Purified DNA for sequencing and cloning was obtained by density gradient ultracentrifugation with CsCl in the presence of ethidium bromide (Sambrook, et al. 1989).

SDS gel electrophoresis was carried out essentially as described by Maniatis. Gels were stained with Coomassie Brilliant Blue R for 30 min at room temperature, then destained overnight. DNA agarous gel electrophoresis was carried out essentially as described by Sambrook, et al. (1989).

Bacterial and yeast strains

Table 3.1 Bacterial and Yeast Strains Used in This Work

E. coli Strain	Genotype
X11-blue	F'::Tn10 *pro*A$^+$B$^+$ *lacI* Δ(*lacZ*)M15/*rec*A1 *end*A1 *gyr*A96(Nal^r) *thi* *hsd*R17($r_K^-m_K^+$) *sup*E44 *rel*A1 *lac*
JM109	F'*tra*D36 *lacI*^{q} Δ(*lac*Z)M15 *pro*A$^+$B$^+$ e14$^-$(*Mcr*A$^-$) Δ(*lac-pro*AB) *thi* *gyr*A96 (Nal^r) *end*A1 *hsd*R17($r_K^-m_K^+$) *rel*A1 *sup*E44 *rec*A1
B121(DE3)	F$^-$*omp*T[*Ion*] *hsdS*$_B$($r_B^-m_B^-$;an *E.coli*B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene

Yeast Strain	Genotype	Reference
15C	MATα, *leu*2, *ura*3-52, Δ*trp*1, *his*4-80, *pep*4-3	generously provided by Professor Andrew Buchman (Penn State University)

Oligonucleotide sequences. The following are the sequences for the primers used in this thesis.

CHMO For - 5'- ATC AAG CAA CCT GCC AAT GCC TAA GCA TGC C -3'

CHMO Rev - 5'- TC GAG GCA TGC TTA GGC ATT GGC AGG TTG CTT GAT -3'

MK1 For - 5'- GGC GGA TCC ATC ATG TCA CAA AAA ATG GAT TTT GAT GC -3'

MK1 Rev - 5'- GCC GCA TGC TTA GGC ATT GGC AGG TTG CTT GAT ATC -3'

3.4 Standard Biotransformations

All biotransformations were completed using the following procedure. Transformed cells are stored at -70°C in 15% glycerol and TE pH 7.5. A small portion of the frozen cells is scraped from the freezer vial using a toothpick and plated on a SC(URA-) plate. This plate is grown at 30°C for approximately 60 hours. One colony is selected to inoculate 25mL of YPD in a 250mL flask. The flask is shaken at 200 rpm at 30°C for 24-26 hours. The culture grows to an OD_{600} of between 4-8. Cells are then harvested by centrifugation at 3000g for 10 min. The cell pellet is then resuspended in 10mL of TE pH 7.5 by vortexing. This procedure is repeated 3

times and the final cell pellet is resuspended at a concentration of 0.1g/mL wet weight cells in TE. At this stage, cells can be added directly to a reaction flask at (0.2g/100mL) or frozen at -70°C for later use. Standard reaction conditions for a preparative biotransformation are 90ml YEP, 10ml 20% galactose stock, 10mM substrate, and 0.2g wet weight cells. One equivalent of β-cyclodextrin can be added if substrate solubility or toxicity is a problem. Reaction flasks are shaken at 30°C (200rpm) and followed by GC. After the biotransformation is complete, the cells are removed by centrifuging at 3000g for 10 min. The aqueous layer is extracted 3 times with an equal volume of ethyl acetate. In addition, the cell pellet is resuspended in 10mL water and extracted with an equal volume of ethyl acetate. The combined organic extracts are dried over magnesium sulfate, filtered and concentrated by rotary evaporation at room temperature. The crude product is purified on a 6in silica column using a 1:1 Hexane/Ether (Still, et al., 1978).

If cells are to be frozen for later use, add glycerol to 15% V/V prior to freezing. Aliquot cells in 0.02g or 0.2g portions per vial. Place immediately in the -70°C freezer. Cells can then be thawed just prior to adding to the reaction flask.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Jon D. Stewart, Chair
Assistant Professor of
Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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May, 1996

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